Phenotype and Transcriptome Responses during Growth and Temperature Stress in the Ciliate *Tetrahymena thermophila*

Dissertation

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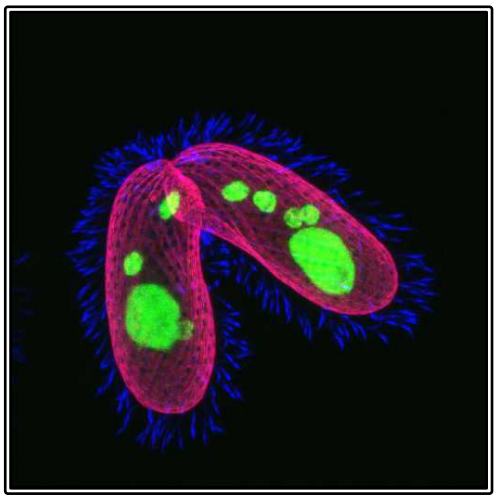
To my family, for their constant support and love, no matter the distance

Contents



Two *Tetrahymena thermophila* cells during conjugation.

Cilia are colored in blue, nuclei are colored in green, cortex is colored in magenta.

Image courtesy of Prof. Eric Stephen Cole, St. Olaf College.

THESIS SUMMARY

Understanding how species respond to biotic and abiotic changes in their environment is an important question in evolutionary biology. This process can be addressed at different levels of biological organization, ranging from molecular up to ecosystem level responses, and integrating these different components provides a greater understanding of the mechanisms that shape species. In this thesis, I investigate phenotypic, transcriptomic and epigenetic responses of a model organism, the ciliate *Tetrahymena thermophila*, exposed to different biotic and abiotic conditions. Temperature is an important abiotic factor and due to the current global change predictions, it is fundamental to understand and predict species responses to temperature change. To explore this question, I exposed populations of *T. thermophila* to a novel temperature close to the species upper thermal limit in a long-term experiment. Significant phenotypic changes were observed, with reduced cell sizes and rounder cells at high temperatures. Transcriptome analyses revealed the importance of energy balance and metabolism repression at high temperatures. To evaluate the stability of the observed phenotypic and transcriptional responses, populations were returned to their original temperature after many generations in the high temperature environment. Morphological traits and also many genes displayed rapid intra-generational changes, indicating plasticity is an important component of this species thermal-stress response. I also investigated phenotypic and transcriptomic patterns during logistic growth, an important stage in the life cycle of this species governed by density dependence. Minor morphological changes were observed during this period, but large gene expression differences were found, mainly between the rapid growth phase and the stationary phase. I also examined the genome-wide distribution of two histone post-translational modifications (PTMs), trimethylation of histone H3 at lysine 4 and acetylation of histone H3 at lysine 9, during the logistic growth phase. Both histone PTMs displayed similar patterns, with enrichments in the exonic regions, marking active genes, but the observed changes in gene expression showed low correlation to changes in the histone PTMs, indicating they are not the major transcriptional modulators acting during population growth. Altogether, exploring the responses of *T. thermophila* to different biotic and abiotic conditions has revealed important mechanisms used by this species when exposed to new environments, corroborating this is a relevant model organism to ecological and evolutionary studies.

Chapter 1. General introduction

Understanding how species respond to changes in biotic and abiotic factors is an important task in ecological and evolutionary research. Abiotic factors include a range of environmental conditions, such as temperature, pH and humidity, and biotic factors include organisms from the same or different species and the many interactions that can occur between them, such as mutualism, competition and predation. In this thesis, I first explore organisms' response to temperature, one of the key abiotic factors affecting all levels of biological organization (Johnston and Bennett 1996). Climate scenarios forecast significant changes in temperature across all ecosystems due to climate change, increasing the need to understand how species respond to novel temperatures (Walther et al. 2002). I also investigate population growth, more specifically logistic dynamics, which is characterized by changes in population density that affect many density-dependent biotic interactions. In both investigations, the ciliate species *Tetrahymena thermophila* is used as a model system.

Species responses to new conditions can be addressed from different perspectives, e.g. molecular responses at the cellular level, physiological or behavioral responses at the individual level, or even population level responses. Integration of these different approaches advances our understanding of the mechanisms that allow species to respond to new environmental conditions (M. E. Feder and Mitchell-Olds 2003). In my doctoral research, I focused on three different response levels. At the molecular level, I monitored gene expression changes, which are a good proxy for the phenotype and are useful to understand cell functioning of microorganisms like *T. thermophila*. Post-translational modifications of proteins were also monitored, in order to evaluate their role in modulating gene expression (Li, Carey, and Workman 2007). At the individual level, I monitored changes in cell morphology, mainly cell size, a key trait in this species (Long and Zufall 2015), and also cell shape. At the population level, population abundance was monitored in all experiments, estimating key demographic parameters such as lag phase duration and growth rate. The integration of these different responses provided a global picture of how this species respond to biotic and abiotic changes in their environment.

Model organisms and the uniqueness of *Tetrahymena thermophila*

Model organisms have played a central role in the development and testing of hypothesis in biology in the last century. They were fundamental in the transformation of early descriptive and observational studies into experimental tests that resulted in a mechanistic understanding of biological processes (Russell et al. 2017). Fast growth and easy manipulation under experimental conditions are important characteristics common to many model species, but the principal benefit of defining and using model organisms is the extensive body of knowledge that is generated about them, allowing scientists to address complex questions and to better interpret experimental results (Müller and Grossniklaus 2010; Ankeny and Leonelli 2011). Important model organisms broadly employed in biological research include the bacterium *Escherichia coli*, the unicellular yeast *Saccharomyces cerevisiae*, the fruitfly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans*, the plant *Arabidopsis thaliana* and the mouse *Mus musculus* (Davis 2004; Müller and Grossniklaus 2010).

Tetrahymena thermophila, a unicellular protist (Figure 1), is commonly listed as a model organism in biology, although it may not be as extensively studied as the previously mentioned organisms. This species is part of the ciliates (Ciliophora), a phylum that forms the Alveolate group together with the dinoflagellates (species with two different flagella that can be phototrophic, predatory, or mixotrophic) and the apicomplexans (mostly parasitic species including *Plasmodium* and *Toxoplasma gondii*). *Paramecium* is the closest relative to *Tetrahymena* that is also considered a model species, although the evolutionary divergence between these two genera is very large, in the order of several hundred million years (Frankel 1999).

Figure 1 Scanning electron micrograph of a *Tetrahymena thermophila* cell. Image credit: Dr. Aaron J. Bell

Important discoveries have been made using *T. thermophila* and exploring many of its unique features. Dyneins were first described in this species, when these proteins were isolated from the abundant cilia present in the cells of this protist (Gibbons and Rowe 1965). The documentation of the catalytic properties of RNA, observed in the self-splicing rRNA of this species (Kruger et al. 1982), resulted in a Nobel Prize to Thomas R. Cech in 1989. This species was also used to identify the repetitive sequences that form the telomere structure and to characterize the telomerase enzyme (Greider and Blackburn 1985; 1989), leading to a Nobel Prize to Elizabeth H. Blackburn, Carol Greider and Jack Szostak in 2009.

As all other ciliates, *T. thermophila* presents the interesting phenomenon of nuclear dualism, i.e. the separation of the germline and somatic functions into two related but separate nuclei, the micronucleus (MIC) and the macronucleus (MAC) (Karrer 2012). The MIC is diploid and only used during sexual reproduction, when it goes through meiosis and takes part in conjugation. The MAC is polyploid and contains the genes that are actively transcribed in the cell. More information about this phenomenon and the general life cycle of this species is shown in Figure 2.

The nuclear dimorphism was very important in early epigenetic studies with *T. thermophila*. Separating the different nuclei, it was possible to identify an enzyme that was enriched only in the MAC and was responsible for the acetylation of histones, characterizing the first histone acetyltransferase (Brownell et al. 1996). The same study also linked histone acetylation to gene activation, leading the way to the investigation of the different histone post-translational modifications and their role in

gene expression regulation, culminating in the “histone code” hypothesis (Jenuwein and Allis 2001).

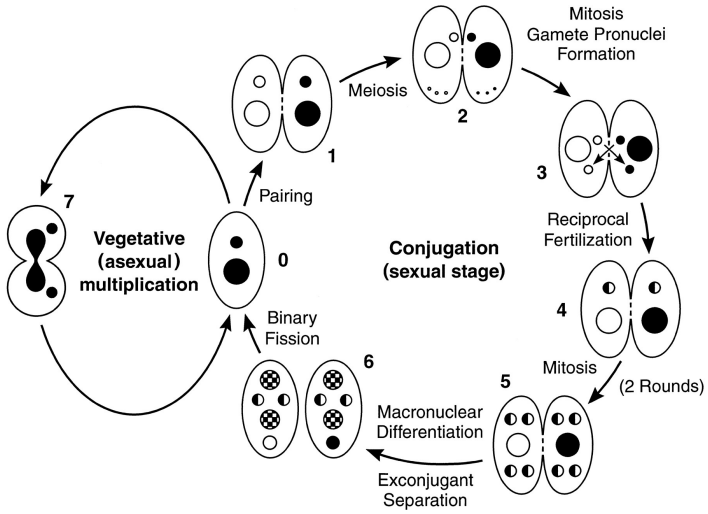


Figure 2 Generalized ciliate life cycle. (*0*) Vegetative cells. Small and large circles represent the micronuclei (MIC) and the macronuclei (MAC), respectively. (*1*) Two paired cells, homozygous for alternative alleles at one locus. (*2*) MICs undergo meiosis, and four haploid nuclei are produced. Only the anterior meiotic product remains functional; the other three disintegrate. (*3*) Mitotic division of functional meiotic product yields genetically identical migratory (anterior) and stationary (posterior) gamete pronuclei. (*4*) Migratory pronuclei are reciprocally exchanged and fuse with stationary pronuclei of the recipient cell, forming the zygote nucleus, which is diploid and, in this instance, heterozygous. (*5*) The zygote nucleus undergoes two mitotic divisions, giving rise to four genetically identical diploid nuclei. (*6*) Two of those nuclei (checkerboard-filled) have differentiated into macronuclei; the other two (solid and white halves) remain diploid micronuclei. The old MACs (at the bottom of each conjugant) are being resorbed and will be lost. This is the stage at which chromosome fragmentation and other site-specific DNA rearrangements occur in the differentiating MAC. The two exconjugants have separated and undergo their first binary fission, restoring the normal nuclear composition (back to stage *0*). (*7*) Vegetative cell dividing by binary fission. The diploid MIC has divided mitotically; the polyploid MAC is undergoing “amitotic division,” pinching off into roughly equal halves. This life cycle scheme is highly conserved among ciliates, although differences of detail occur in particular groups and species. Figure and legend adapted from (Orias 1998)

Another interesting feature of the life cycle of *T. thermophila* is its unique reproduction mode. In favorable conditions, cells grow and then divide through binary fission, but when environmental conditions become stressful, mainly due to nutrient limitation, cells undergo sexual reproduction through conjugation (Figure 2). *T.*

thermophila presents seven different mating types, and conjugation only takes place between cells that belong to different mating types. Interestingly, when two cells mate, the resulting progeny can present any of the seven existent mating types. The fascinating mechanism that this process type was recently described and involves two genes in the mating type locus that go through programmed DNA rearrangements, resulting in a system with stochastic mating type determination (Cervantes et al. 2013).

DNA rearrangements have also a central role in nuclei development after conjugation. Although the two nuclei inside each cell are related, since the MAC originates from the MIC, they differ in ploidy; while the MIC is diploid and consists of five chromosomes, the MAC is polyploid (~45 N) and composed of approximately 225 chromosomes. This difference in ploidy is caused by genome-wide DNA rearrangements that take place upon MAC formation. Programmed chromosome breakage and massive DNA elimination occurs, removing repetitive sequences, introns and intergenic regions, which results in the elimination of one third of the MIC genome (Karrer 2012; Hamilton et al. 2016). Both nuclei of *T. thermophila* have been fully sequenced (Eisen et al. 2006; Hamilton et al. 2016), preparing the entry of this model system into the genomics era.

The genomics revolution

The first complete sequence of a genome was published only 25 years ago, when the small genome of the virus *Haemophilus influenza* was fully decoded (Fleischmann et al. 1995). A few years later, in the early 2000s, the human genome project completed the sequence of the first human genome and also gave rise to the development of the high-throughput sequencing technologies currently used. These new technologies enabled the generation of unprecedented amounts of data at continuously decreasing costs, making genomic tools available to all research areas. DNA sequencing was quickly followed by the sequencing of related molecules, opening up the new fields of transcriptomics, epigenomics, metabolomics and others. The latest developments allow researchers to sequence single cells individually, providing an extraordinary level of detail to the obtained molecular data (Shapiro, Biezuner, and Linnarsson 2013).

Ecological and evolutionary studies have been greatly advanced by the genomics revolution. Experimental evolution, for example, is now able to track mutations across the entire genome, detecting when they occur and how their frequencies change through time (e.g. Blount et al. 2012). Speciation and adaptation studies have also seen great progress with the use of genomic data, allowing a better understanding of processes such as gene flow, hybridization and the importance of genome architecture in these evolutionary processes (J. L. Feder, Egan, and Nosil 2012; Semenov et al. 2019). Natural communities can be assessed through metagenomics, a technique that uses high-throughput sequencing of genetic material obtained from environmental samples for taxa identification and has large potential for species conservation and monitoring projects (e.g. Yu et al. 2012; Gibson et al. 2015). The genomic revolution reaches as far as paleontology, with the sequencing of historical and ancient samples of a diverse range of organisms, from viruses (Matthieu Legendre et al. 2014) to Neanderthals (Noonan et al. 2006).

Besides the sequencing of the macronuclear and micronuclear genomes of *T. thermophila*, other studies have also used genomic tools to investigate different processes in this ciliate. The transcriptome of *T. thermophila* was sequenced during its main life cycle stages (Xiong et al. 2012) and in knockout experiments to evaluate the role of different genes (Xu et al. 2016; Yan et al. 2016). Nucleosome positioning (Luo et al. 2018) and N^6-methyldeoxyadenosine (Wang et al. 2017; Luo et al. 2018) were also investigated with high-throughput sequencing technologies, advancing the knowledge of the chromatin structure of this species. Recently, single-cell whole-genome sequencing was successfully employed in the identification of single nucleotide polymorphisms in *T. thermophila* (Chen et al. 2019).

Thesis objective and outline

My doctoral research consisted of two main projects with the ciliate *T. thermophila*. The first project aimed at exploring how a novel high temperature affects phenotypic traits and transcriptome dynamics in this ciliate. This environmental condition was chosen due to its fundamental importance in shaping all biological processes. Although *T. thermophila* can grow in temperatures as high as 40 °C, little is known about the molecular basis of its temperature response. I first explore the strong phenotypic effects that exposure to high temperature produced in

the cell morphology of this species, which comprises **Chapter 2**. The functional genomics data from the same temperature experiment is then investigated in **Chapter 3**, revealing important molecular mechanisms that allow survival at such stressful condition.

The second experiment of my doctoral project explores the genetic basis of population growth and the role of two histone post-translational modifications (PTMs), the trimethylation of histone H3 at lysine 4 (H3K4me3) and acetylation of histone H3 at lysine 9 (H3K9ac), in this process. The results of this investigation can be seen in **Chapter 4**. The final Chapter of this thesis is a general discussion of the research using *T. thermophila* as a model system and summarizes the main findings of this thesis, including future directions to further advance these projects.

General methods

The *T. thermophila* strain 1630/1U was used in all experimental work. Since only one mating type was present in the populations, only clonal reproduction took place. The stock cultures used to start the experiments underwent long acclimation periods to the conditions in our laboratory and to the proteose peptone growth medium used in all experiments. All populations were grown in axenic conditions, using autoclaved medium. This strain has a long history of serial transfers, and although stock populations were not initiated from a single individual, we believe genetic variation in the starting populations of the experiments was very low (Ketola et al. 2004).

In all experiments, population abundances and cell morphology data were obtained through videos of small samples of the *T. thermophila* populations. I used the R package BEMOVI (Pennekamp, Schtickzelle, and Petchey 2015) to identify the moving cells in the videos and extract morphological information from all the detected cells. This method is extremely fast and efficient, producing high quality data which greatly improved the monitoring of experimental populations and facilitated the execution of large-scale experiments.

Two genomic methods were employed in this thesis. Whole transcriptome sequencing (RNA-seq) was used in the temperature and in the growth experiments to assess all genes transcribed in the experimental populations. Chromatin immunoprecipitation sequencing (ChIP-seq) was used in the growth experiment to

assess the genome-wide distribution of the two histone PTMs, H3K4me3 and H3K9ac.

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Chapter 2. Phenotypic responses to temperature in the ciliate *Tetrahymena thermophila*

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Under revision in *Ecology and Evolution*

Abstract

Understanding the effects of temperature on ecological and evolutionary processes is crucial for generating future climate adaptation scenarios. Using experimental evolution, we evolved the model ciliate *Tetrahymena thermophila* in an initially novel high temperature environment for more than 35 generations, closely monitoring population dynamics and morphological changes. We observed long lag phases, a strong reduction in cell size and modifications in cell shape at high temperature. When exposing the adapted populations to their original temperature, most phenotypic traits returned to the observed levels in the ancestral populations, indicating phenotypic plasticity is an important component of this species thermal stress response. However, persistent changes in cell size were detected, indicating possible costs related to the adaptation process. Exploring the molecular basis of thermal adaptation will help clarify the mechanisms driving these phenotypic responses.

Keywords

Temperature, experimental evolution, adaptation, morphology, *Tetrahymena*

1. Introduction

Temperature is one of the most important abiotic factors, influencing all levels of biological organization, from cell function to ecosystem dynamics (Johnston and Bennett 1996). Understanding how organisms respond and adapt to a novel temperature has, therefore, been the focus of multiple studies exploring physiological, ecological and evolutionary mechanisms (Angilletta 2009; Clarke 2003). The current climate change crisis revived the interest in this research field, since understanding how populations will respond to new temperatures is of fundamental importance (Walther et al. 2002; Hoffmann and Sgrò 2011).

Microorganisms, like many other ectotherms, are particularly sensitive to the temperature of their environment as it directly affects their metabolism and many physiological processes (Pörtner et al. 2006). Since microorganisms play key functions in all ecosystems, understanding their responses to temperature is essential to forecasting the future of ecosystems (Singh et al. 2010). Besides their ecological importance, many of these organisms have short life cycles, large population sizes and are readily manipulated in the laboratory, offering many possibilities to experimentally study thermal adaptation over multiple generations (Elena and Lenski 2003; McDonald 2019).

The effect of temperature on the size of microorganisms is one of the most studied morphological responses to temperature. Most species display smaller cell sizes when grown at higher temperatures, a response known as the temperature-size rule (Atkinson 1994). Besides cell size, many important phenotypic traits such as cell shape (Trueba et al. 1982) and swimming behavior are also affected by temperature (Schneider and Doetsch 1977; Beveridge, Petchey, and Humphries 2010).

Organisms can use different mechanisms to survive in a novel temperature. Many species exhibit phenotypic plasticity, i.e. the same genotype can generate multiple phenotypes. Plastic responses can be further separated into two different types: developmental plasticity, i.e. traits that vary according to the environment of the development, but are then irreversible during an organism's life spam; or traits that are context-dependent and show variation in the same individual, such as behavior or metabolic reactions, sometimes called phenotypic flexibility (Piersma and Drent 2003). Acclimatization, the adjustment of physiological traits to environmental conditions, is one example of phenotypic flexibility (Wilson and Franklin 2002;

Piersma and Drent 2003). Species can also adapt to a novel temperature, which occurs when genetic changes lead to a population with higher fitness in the new environment. Temperature adaptation can lead to the evolution of specialization and have costs to an organism, such as reduced performance in the ancestral or other environments (Huey and Kingsolver 1989). These costs are predicted by theory and have been observed in previous experiments (Bennett and Lenski 2007; Jin and Agustí 2018). Phenotypic plasticity and adaptation are not mutually exclusive mechanisms, in fact it is likely that they are combined in many responses to environmental change (Davis and Shaw 2001; Philip Gienapp et al. 2008).

Several studies have experimentally examined thermal adaptation in microorganisms such as bacteria (Trueba et al. 1982; Hall, Neuhauser, and Cotner 2008; Tenaillon et al. 2012; Sandberg et al. 2014), phytoplankton (Schlüter et al. 2014; Padfield et al. 2016) and yeast (Caspeta et al. 2014; Huang et al. 2018), but this topic has been little explored in protists. To better understand temperature adaptation in this group, we chose the ciliate protist *Tetrahymena thermophila* as our model system. This is one of the best-studied species of protists and it is able to grow in a wide range of temperatures, it is thus a suitable organism for our experimental evolution approach.

In this study, we monitored the population dynamics and cell morphology of four replicate populations of *T. thermophila* exposed to 38 °C, a highly stressful condition. With this, we tested whether populations can survive in a temperature near lethality without previous acclimation. Different mechanism can be involved in this process, from phenotypic plasticity to adaptation to the new environment. When exposed to such a high temperature, we predict the growth rate of *T. thermophila* populations will have an immediate and strong decrease. This prediction is based on the observed reductions of population growth at temperatures above 37.5 °C (see Figure 1, which contains results from a pilot experiment described in the Methods section), and also because this temperature is close to the thermal limit of the species (Laakso, Löytynoja, and Kaitala 2003).

We hypothesize a negative effect of temperature on cell size, as predicted by the temperature-size rule (Atkinson 1994) and established for many protists (Atkinson, Ciotti, and Montagnes 2003). The shape of protist cells can be affected by stressful environmental conditions (Kovács et al. 1999; Dias, Mortara, and Lima 2003), by the presence of predators (Kuhlmann and Heckmann 1985; Hammill, Petchey, and Anholt 2010) and is related to dispersal behavior (Pennekamp et al.

2014). Absent, however, are clear hypotheses about how and why cell shape would change with temperature. Nevertheless, we present analyses of the effects of temperature on cell shape.

After many generations at this novel temperature, the populations were then returned to the control temperature, a benign environment. This enabled us to test if survival at high temperatures had any costs to the organisms. If there are costs associated to survival at high temperature, we expect to see reduced growth rates when adapted populations return to the control condition. Similar patterns should also be observed for the morphological traits, with reduced cell sizes when populations return to the control condition. Opposite results would indicate the evolution of generalists, or the presence of a plastic response.

2. Materials and methods

2.1. Strain and culture conditions

All experiments were performed with the ciliate *Tetrahymena thermophila* (Figure 2A) strain 1630/1U cultured in axenic conditions in 2 % proteose peptone medium. This strain was acquired from the Culture Collection of Algae and Protozoa and grown during many generations at 15 °C to acclimate it to our laboratory conditions. We did not initiate the stock cultures from single cells since this strain likely presents a reduced genetic variability due to a long history of serial transfers (Ketola et al. 2004). *T. thermophila* only reproduced clonally in all experiments, since a single strain with one mating type was used.

The medium used in all experiments was prepared with proteose peptone from the same manufacturer batch, ensuring homogeneous conditions across all experimental replicates. The bottles and the medium used in the experiments were sterilized in an autoclave and all sampling procedures were performed in sterile conditions. Microbial contamination was regularly checked during the experiments by plating a sample of each culture on an agar plate incubated at 37 °C for 24 h. To ensure our treatments were reliable, we monitored every 15 minutes the actual temperature in the incubators used to grow the experimental populations. In all

incubators, the mean daily temperature presented a standard deviation smaller than 0.28 °C.

2.2. Temperature range of *T. thermophila*

We performed a pilot experiment to identify the temperature range in which this *T. thermophila* strain is able to grow, exploring ten different temperatures from 20 to 42.5 °C, in intervals of 2.5 °C. Three replicate populations were grown in each temperature for a period of 13 days. The initial population density of all replicates was 500 cells/ml and the populations were monitored daily with videos to measure population density.

We observed cell division in all tested temperatures except at 42.5 °C (Figure 1). When grown in temperatures between 20 and 37.5 °C, populations immediately entered exponential growth and reached high and stable carrying capacities. At 40 °C, populations initially increased in density but collapsed after two days, indicating this temperature is close to the upper thermal limit of this strain.

To explore temperature adaptation in this species, we chose 20 °C as the control temperature of the experiment and 38 °C as the adaptation temperature. At 20 °C, populations grow very well and reach carrying capacity in a short period of time, while 38 °C is a stressful temperature that creates a strong selective pressure.

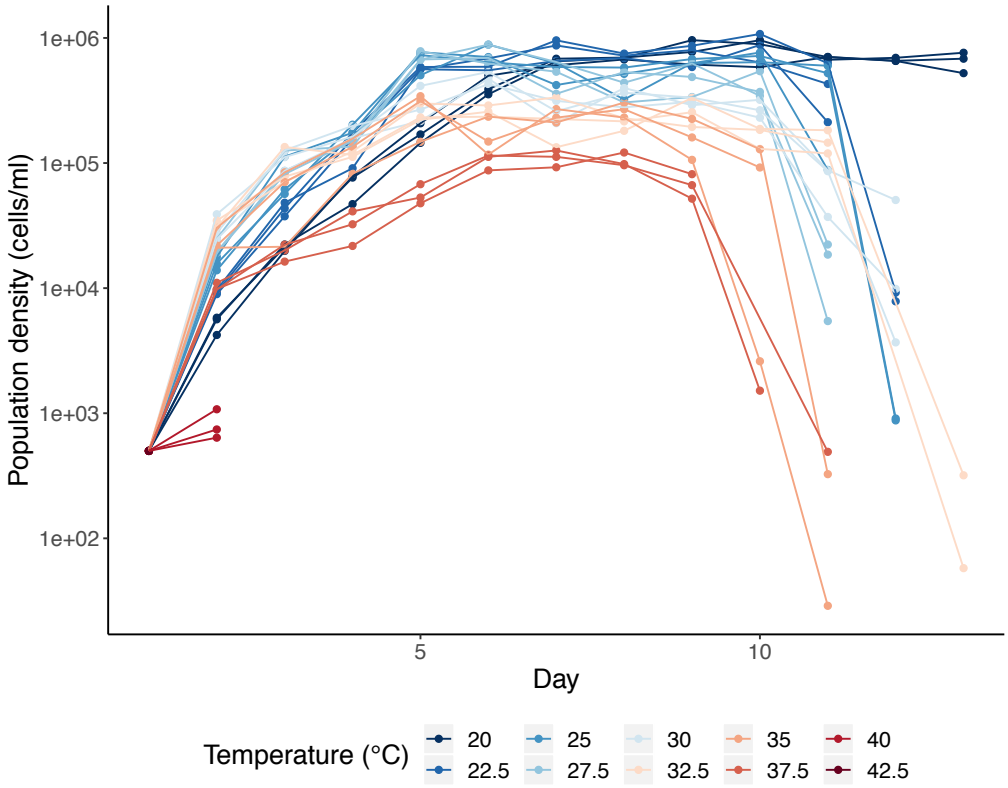

Figure 1 Population dynamics of *T. thermophila* strain 1630/1U growing in ten different temperatures. Each line represents one replicate population and the colors indicate the temperature in which the population was grown.

2.3. Experimental design

The temperature adaptation experiment was performed with populations of *T. thermophila* growing in axenic batch cultures in 2-liter bottles with 500 ml of medium. The cultures were placed in incubators with controlled temperature, no light and in shakers to increase aeration. The experiment was initiated with four replicate populations from the same stock culture, giving rise to four separate evolving lineages. There were five consecutive batch cultures of each lineage that lasted a total of 41 days (Figure 2).

All the batch cultures started at a low density (500 cells/ml) and once they reached carrying capacity new batch cultures were started from a small aliquot of the

previous batch. The volume of this transferred aliquot depended on the population density of the culture at the end of the previous batch, to ensure that all new cultures started with the same cell density (500 cells/ml). Each batch had a different duration, since the time to reach carrying capacity changed during the experiment, in large part due to the temperature treatment. The shortest batch lasted five days, while the longest batch lasted 12 days.

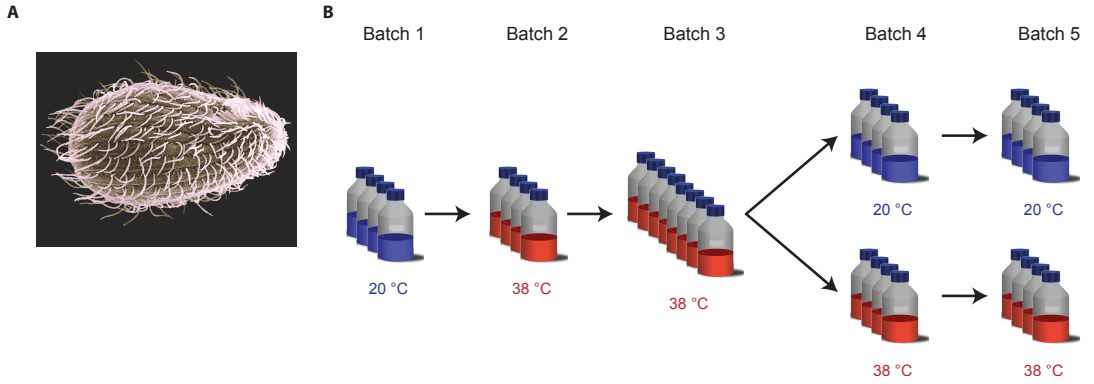

Figure 2 Image of the ciliate *Tetrahymena thermophila* (A) and experimental design of the temperature experiment (B). In the schematic of the experiment, each bottle represents one replicate batch culture, and the colors indicate the temperature in which the culture was grown. (A) Image credit: Dr. Aaron J. Bell.

In the first batch, the four replicate cultures were grown at 20 °C and in the second batch, the four replicates started to grow at 38 °C. Each culture in the second batch originated two cultures in the third batch, resulting in eight cultures still growing at 38 °C. In the fourth batch, one of each paired culture was moved back to 20 °C, while the other culture remained at 38 °C. In batch five, the cultures continued in the temperature experienced in the previous batch.

2.4. Video monitoring and processing

The cultures were monitored daily after the second or the third day of each batch, and every day in batch 5. These minor differences in the monitoring schedule compensated for minor differences in the timing of population dynamics. Monitoring included estimation of population abundances and different morphological measurements. On the monitoring days, each culture was sampled twice, since duplicate assessments provide more accurate estimates of the population abundance. Each sample consisted of 1 ml of culture. The samples were placed in counting chambers and the videos were taken on a stereomicroscope (Leica M205 C) mounted with a digital CMOS camera (Hamamatsu Orca C11440, Hamamatsu Photonics, Japan) with 1.57X magnification. When population density was high, samples were diluted with fresh medium before taking the videos. Each video comprised 125 frames in 5 seconds and monitored 40.26 µl of sample. The videos were processed using the R package BEMOVI (Pennekamp, Schtickzelle, and Petchey 2015), which extracts morphological information of all the moving cells in the field of view.

2.5. Data analysis

In total, 94,344 cells were measured during the experiment, with an average of 513 ± 26.9 cells monitored per population per day. The number of detected cells was used to estimate population density throughout the experiment. We calculated the minimum number of generations (G) that took place in each batch culture with the equation $G = \ln(A_{max}/A_0)/\ln(2)$, where A_0 is the minimum population abundance, and A_{max} is the maximum population abundance.

All statistical analyses were performed using R (R Core Team 2019). We used a Gompertz model (Marcel H. Zwietering et al. 1990) implemented in the R package growthrates version 0.8.1 (Petzoldt 2019) to estimate the maximum growth rate and the duration of the lag phase in each population per batch. The model fittings can be seen in Figure S1. Despite different optimization strategies, this model presented low R^2 values for population 3 in batch 4 at 38 °C, indicating it could not properly model the growth dynamics of this population. We therefore calculated the maximum growth rate of this population as the $\log_{10}$ difference in abundance between

the maximum and the minimum population abundance, divided by the time period between these days. The lag phase of this population was set to zero, since it immediately entered exponential growth.

We used two morphological measurements in this study, the cell size and the cell shape (the ratio between the longer and the shorter axes of the cell). The morphological measurements were averaged per population per day and the coefficient of variation among individuals for cell size was calculated per population per day. We recorded information related to movement behaviour, such as swimming speed, but did not include this data in the present study. We acquired videos at room temperature, which differed from the growing temperature, and some samples for videoing required dilution due to high density. Therefore, we are not confident that movement behaviours recorded from the videos would reflect effects of the temperature treatments of the experiment.

To investigate the effect of temperature on population dynamics and cell morphology, we performed two separate analyses, first on the populations at 38 °C and then on the populations that returned to the control temperature. We analyzed six different traits, the population growth rate, the lag phase duration, the mean cell size, its coefficients of variation and its variance, and the mean cell shape. For each of these six response variables, the average change relative to the control (batch 1) was calculated for each population in each batch.

We used the package MCMCglmm version 2.29 (Hadfield 2010) to fit linear mixed effects models to each of the five response variables. All models included population lineage as a random effect and time as a fixed effect. We ran each model for 2,000,000 iterations, with a burn in of 30,000 iterations and storing every 1,000th iteration. We assessed model convergence with autocorrelation analyses and with trace plots using the package coda version 0.19-3 (Plummer et al. 2006).

3. Results

3.1. Population dynamics

All replicate populations grew well in the control temperature of 20 °C in the first batch (Figure 3A), immediately entering exponential phase and reaching carrying capacity in a few days. When populations started to grow at 38 °C, they entered a long lag phase and exponential growth started only after 7 days (Figure 4B). Similar dynamics were observed in batch 3, even though a few populations displayed a shorter lag phase of 3 to 4 days. In batch 4, the populations that continued at 38 °C displayed a reduced lag phase, similar to the populations that went back to the control temperature. Batch 5 had similar population dynamics to what was observed in the previous batch. The four batches in which *T. thermophila* populations experienced 38 °C comprise a minimum of 35 generations.

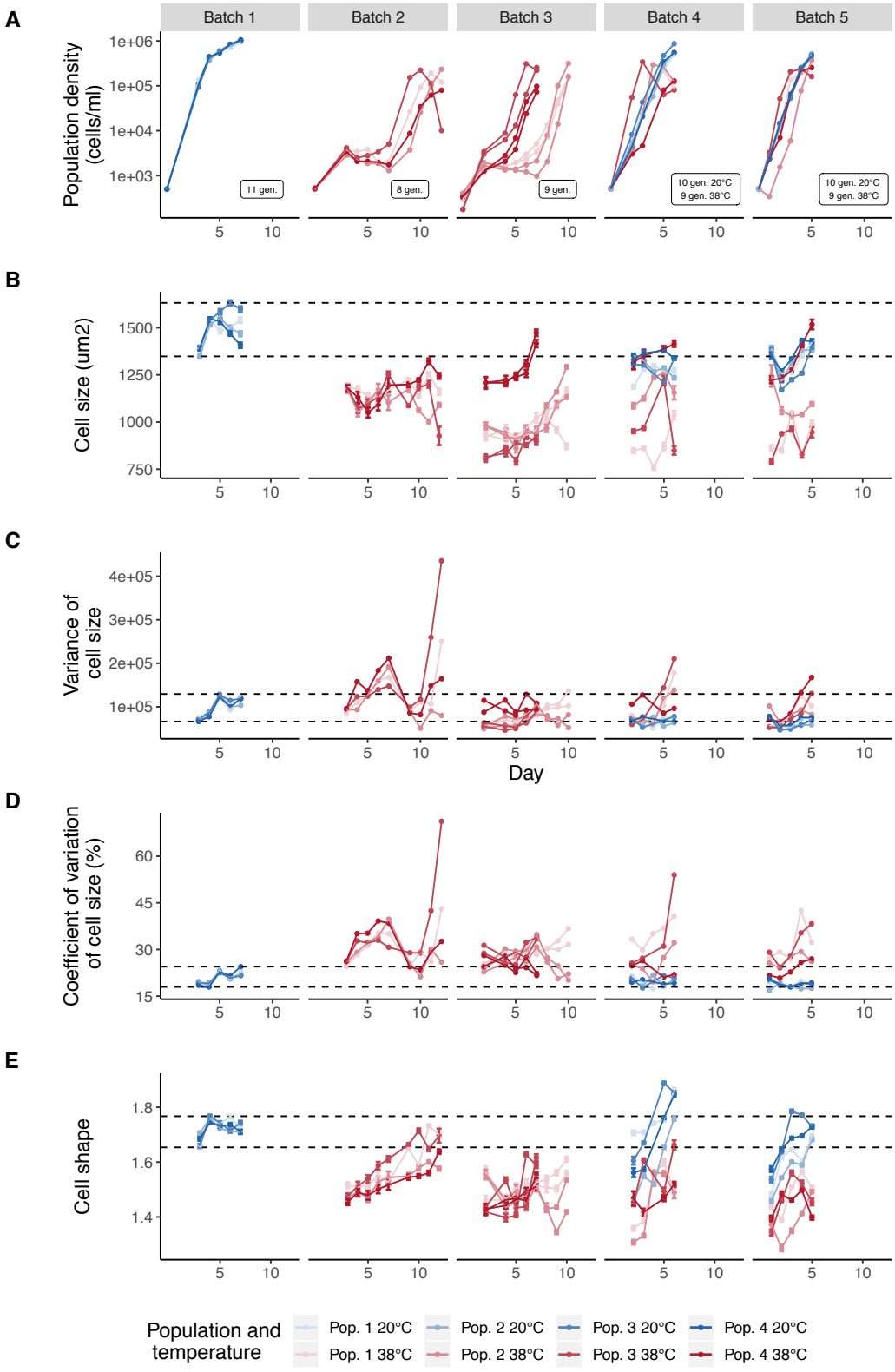

Figure 3 Population dynamics and morphological traits of each *T. thermophila* population during the temperature experiment. Population density (A), mean cell size (B), variance of cell size (C),

coefficient of variation of cell size (D) and mean cell shape (E) are shown for each population and for each batch separately. Minimum number of generations that took place in each batch is shown in boxes in plot A. Error bars indicate standard errors of means for cell size and cell shape (B and E). The colors indicate the temperature in which the population was grown, and the shades represent the population replicate. Dashed lines mark the range of observed values at the control temperature (20 °C) in the first batch of the experiment.

Temperature strongly affected the lag phase of *T. thermophila* cultures. In the first batch at 38 °C, lag phase was increased by 7.7 days [6.3; 8.9] (here and later in square brackets is the estimated 95% credible interval) in comparison to the control temperature, but it gradually decreased during the experiment. In the final batch, lag phase at 38 °C was much shorter (0.7 days [-0.6; 2.1]) and returned to control levels (Figure 4B and Figure 5).

Maximum growth rate slightly increased during the adaptation to 38 °C, and although we observed a large variation between the population lineages, we still found a significant effect of temperature, with an increase of 4.5 [0.4; 8.7] at the end of batch 5 (Figure 4B and Figure 5). Maximum population density decreased at 38 °C, but it remained lower throughout all batches (Figure 3A). In batch 4, the populations that moved back to 20 °C displayed an immediate increase in maximum population density, similar to the density observed in batch 1.

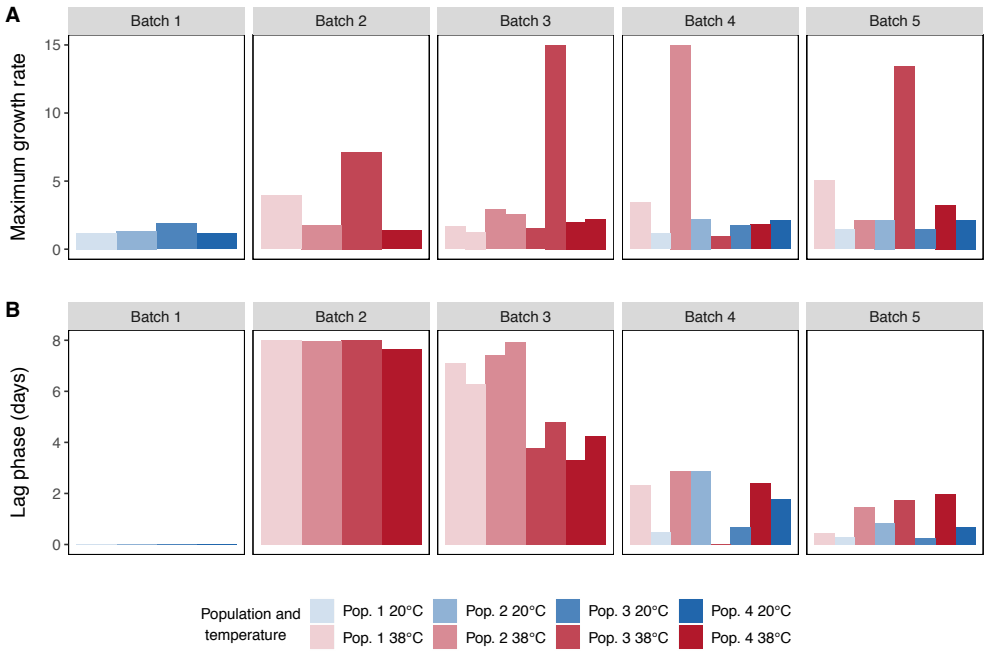

Figure 4 Maximum growth rate (A) and lag phase (B) of each *T. thermophila* population per batch culture estimated with the Gompertz model. The colors indicate the temperature in which the population was grown, and the shades represent the population replicate.

3.2. Cell morphology

Temperature had a strong and long-lasting effect on cell size and shape. There was an immediate decrease of 27.3 % [9.3; 47.5] in cell area when populations moved to the higher temperature (Figure 3B and Figure 5). This size reduction was maintained throughout the entire experiment and populations displayed a cell area reduction of 30.2 % [11.2; 49.8] at the final batch. When populations moved back to 20 °C after many generations growing at 38 °C, cell size recovered and the mean cell area increased, but populations did not return to the initial conditions observed in batch 1 (-11.1 %, [-16.6; -5.0]).

To explore the variation in cell sizes during the experiment, we modeled both the variance and the coefficient of variation (CV) of cell size in response to temperature, which showed slightly different patterns, since the CV is scaled by the sample mean. The CV of cell area significantly increased at 38 °C through the entire experiment (32.9 % [5.75; 56.6] in batch 5) (Figure 3D and Figure 5), while the variance in cell size was not different than the control (-17.4 % [-39.5; 4.79] in batch

5) (Figure 3C and Figure 5). Taken together, they indicate a slightly wider range of cell sizes when populations first move to the novel temperature, but a reduction in variation towards the end of the experiment (Figure 5).

Similar patterns were observed in cell shape, as can be seen in the mean cell shape of the populations during the temperature experiment (Figure 3E and Figure 5). Cells became rounder as they adapted to 38 °C (16.5 % [14.1; 19.0] rounder in batch 5), and cell shape returned to more elongated forms when populations moved back to 20 °C (5.2 % [0.1; 10.6] rounder in batch 5).

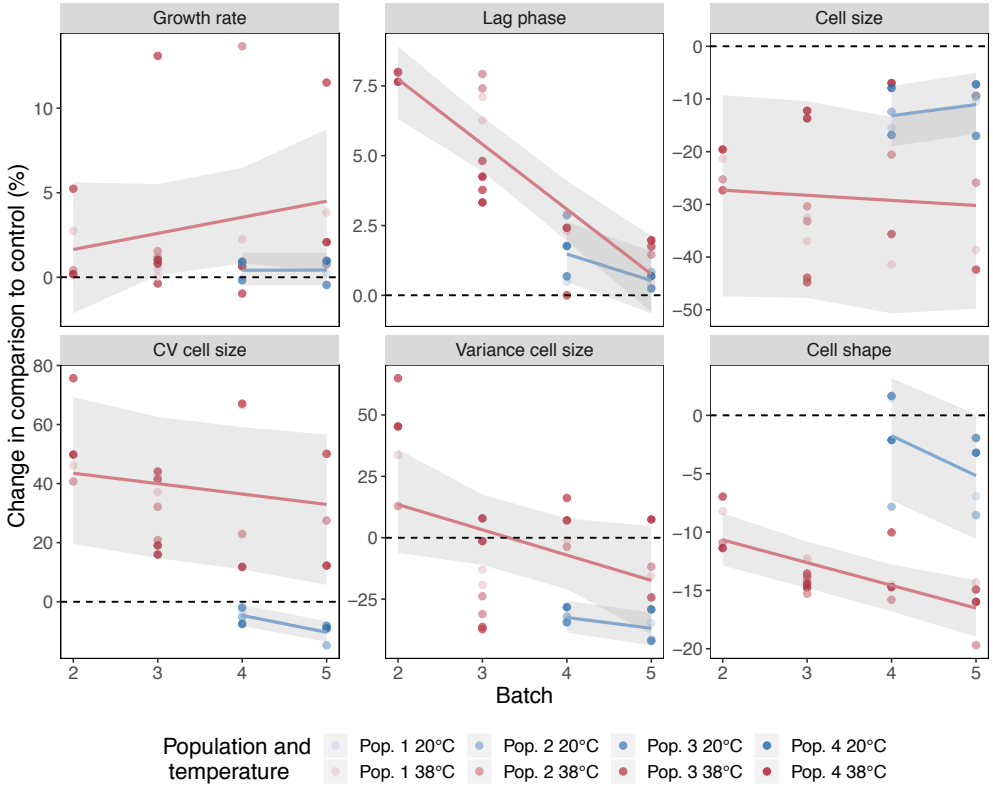

Figure 5 Change in population dynamics and morphological traits of *T. thermophila* populations. Change in maximum growth rate, lag phase, cell size, coefficient of variation (CV) of cell size, variance of cell size and cell shape are shown, for each population and for each batch separately, using the mean of the control cultures at 20 °C in batch 1 as a reference. Maximum growth rate difference is expressed in day^{-1} and lag phase difference is expressed in days, while all other values are expressed as percent difference. The lines represent the fitted mixed effects models and the shaded areas represent the 95 % credible interval (see methods for details). The colors indicate the temperature in which the population was grown, and the shades represent the population replicate. The dashed lines mark no change in comparison to the control.

4. Discussion

The results of this experiment clarify phenotypic and population responses of *T. thermophila* exposed to a high temperature environment for more than 35 generations. As hypothesized, population dynamics were strongly affected by the high temperature, although we observed a strong negative effect on the lag phase and not on the growth rate of the populations. The long lag phases in the first batch at 38 °C returned to control levels after around 26 generations, while growth rate at 38 °C slightly increased during the experiment.

Phenotypic effects were observed in the morphology of *T. thermophila*, with a prevalence of smaller and rounder cells at high temperature. Previous experiments using *Tetrahymena* species have also observed reductions in cell size at high temperatures (James and Read 1957; DeLong et al. 2017), a pattern also present in other ciliates (e.g., Weisse et al. 2002), but the duration of these experiments was much shorter, comprising only a small number of generations. Our study shows that cell size is immediately reduced at high temperatures and remains lower as populations evolve. Despite this general trend, one of the replicate populations displayed a much smaller cell size reduction (population 4, Figure 3), indicating that larger cells are also a viable phenotype at high temperatures. Furthermore, cell size showed an increased variation at the high temperature environment, while at 20 °C cells had a more uniform morphology. Stressful conditions often lead to a higher phenotypic variability (Hoffmann and Hercus 2000), and this pattern could be another indication that more than one phenotype is viable in this environmental condition.

Different hypotheses try to clarify the mechanisms through which temperature affects body size. Although most of them were developed to explain the plastic response of the temperature-size rule, these hypotheses are also applicable for long-term adaptive responses to temperature. One hypothesis relates smaller cells at high temperatures to higher metabolic rates and therefore higher oxygen demands. Since oxygen diffusion is reduced as temperature increases, a reduction in cell size compensates for that (Atkinson, Morley, and Hughes 2006). Another possible explanation is based on growth rate being more affected by temperature than development rate, which would lead to organisms dividing at a younger age and thus being smaller (Zuo et al. 2012). A third mechanism is based on body size optimization between the organisms' demand and their expected resource supply in

a given temperature (DeLong 2012). These hypotheses are not mutually exclusive, and all are relevant to our study system. Each of these hypotheses received support from theoretical models and experimental data, but their importance for the observed patterns is still under debate.

Little is known about the effect of temperature on cell shape in *T. thermophila* and in other ciliates. DeLong et al. (2017) grew *T. thermophila* in three different temperatures, 20, 26 and 32 °C, and although variation in cell shape was observed, no clear pattern related to temperature was found. A few studies exposed populations of *T. thermophila* to different stressful conditions and have also observed rounder cells (Dias, Mortara, and Lima 2003; Nilsson 2005). Taken together, these experiments indicate that round cells could be related to harsh environmental conditions in general, and not only to high temperature. The rounder cells could also be connected to malfunction of cytoskeleton proteins at high temperature, since these proteins have an important role in maintaining cell shape (Williams 2004). Investigations of gene and protein functions in high temperature environments would help clarify these mechanisms.

Possible costs related to thermal adaptation were estimated by analyzing the performance of the populations that returned to the control temperature after more than 18 generations at 38 °C. No significant reduction was observed at the growth rate of batches 4 and 5, and cell shape returned to the control levels, indicating little costs related to the high temperature adaptation. Cell size, however, remained smaller even after many generations back in the control temperature (Figure 5), and populations displayed a small increase in lag phase during batch 4 at 20 °C (Figure 5). Cell size is an important trait for the fitness of unicellular organisms (Monds et al. 2014) including *T. thermophila* (Long and Zufall 2015) and the observed pattern may indicate the occurrence of costs when populations adapt to a new temperature.

Evidence for costs related to thermal adaptation have been described in previous experiments investigating different microorganisms. Bennett and Lenski (2007) found fitness trade-offs in *E. coli* populations adapted to 20 °C in comparison to the ancestral populations adapted to 40 °C. Baker et al. (2018) described trade-offs in growth rate of a dinoflagellate adapted to supra-optimal temperature, and Duncan et al. (2011) , using another ciliate, *Paramecium caudatum*, observed trade-offs when populations were adapted to a specific temperature and became specialists. The dynamics of cell size found in this study indicate that costs may also take place in our study system, but longer experiments are needed to confirm the

relevance of this, since trade-offs might be transient and only present while populations are still adapting to the new environment.

Phenotypic plasticity can also play an important role in thermal adaptation, as observed in experiments with bacteria (Shi and Xia 2003) and zooplankton (Yampolsky, Schaer, and Ebert 2013). A plastic response likely also explains some of the patterns in this study, for example the immediate recover of cell shape when populations return to 20 °C in batches 4 and 5 (Figure 4). The long lag phases observed in the batch 2 (Figure 4B), the first batch exposed to the novel temperature, probably also represents a plastic response in the form of acclimation to the new environment. However, populations also displayed longer lag phases in batch 3, and acclimation is not sufficient to explain this pattern, since populations had already been exposed to this high temperature for multiple generations. Developmental plasticity and adaptive responses are possible mechanisms generating the observed temperature response. Analysis of the molecular basis of this response could clarify the role of plasticity and would also help understanding the mechanisms behind the phenotypic changes during thermal adaptation.

One important feature of our study, caused by time and resource constraints, was the use of the ancestral populations grown at 20 °C in batch 1 as the control (Figure 2), instead of maintaining populations at 20 °C during the entire experiment, or keeping individuals from batch 1 in suspended animation (which is technically difficult for *T. thermophila*) to compare with individuals from later batches in a common garden setting. When comparing the ancestral populations in batch 1 (20 °C) and the evolved populations in batch 5 (20 °C), as we did, two things differ: 1) prior exposure to 38 °C (our treatment) and amount of time in the experimental conditions (i.e. batch). Although we cannot rule out the possibility, we find it very unlikely that the amount of time in experimental conditions could account for the observed differences because: i) individuals in batch 1 had already experienced many generations in growing conditions similar to the experimental conditions; ii) we observe low variation in population dynamics and morphological traits, relative to treatment effects; iii) comparison with previous 20 °C populations from the pilot experiment (not shown).

Previous studies found extensive variation of dispersal propensity across different genotypes of *T. thermophila* (Pennekamp et al. 2014), so different thermal tolerances could also be expected for this species. It would therefore be relevant to investigate if different *T. thermophila* strains display growth and morphological

responses to high temperature similar to the ones we observed in this experiment. Patterns of temperature adaptation in natural populations of *T. thermophila* or other ciliates have received little attention so far. Krenek, Petzoldt, and Berendonk (2012) explored this question in *Paramecium caudatum* populations sampled in a latitudinal range across Europe but found no indication of local adaptation in this species. Our study only investigates a single strain of *T. thermophila*, but the results provide evidence that generalists are present in this species as well, since adaptation to a higher temperature had little effect on growth at the ancestral temperature. Studying natural populations of this species would be an interesting comparison to the results obtained in these laboratory experiments and would help better understand the process of thermal adaptation in microorganisms.

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Author contributions

VWM and OP designed the experiments and wrote the manuscript. VWM performed the experiments and analyzed the data, with input from OP.

Data accessibility

Population abundances and morphological trait data will be available upon publication on Dryad.

Scripts for all figures and statistical analyses will be publicly available upon publication.

Competing interests

The authors declare no competing interests.

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Supplementary material

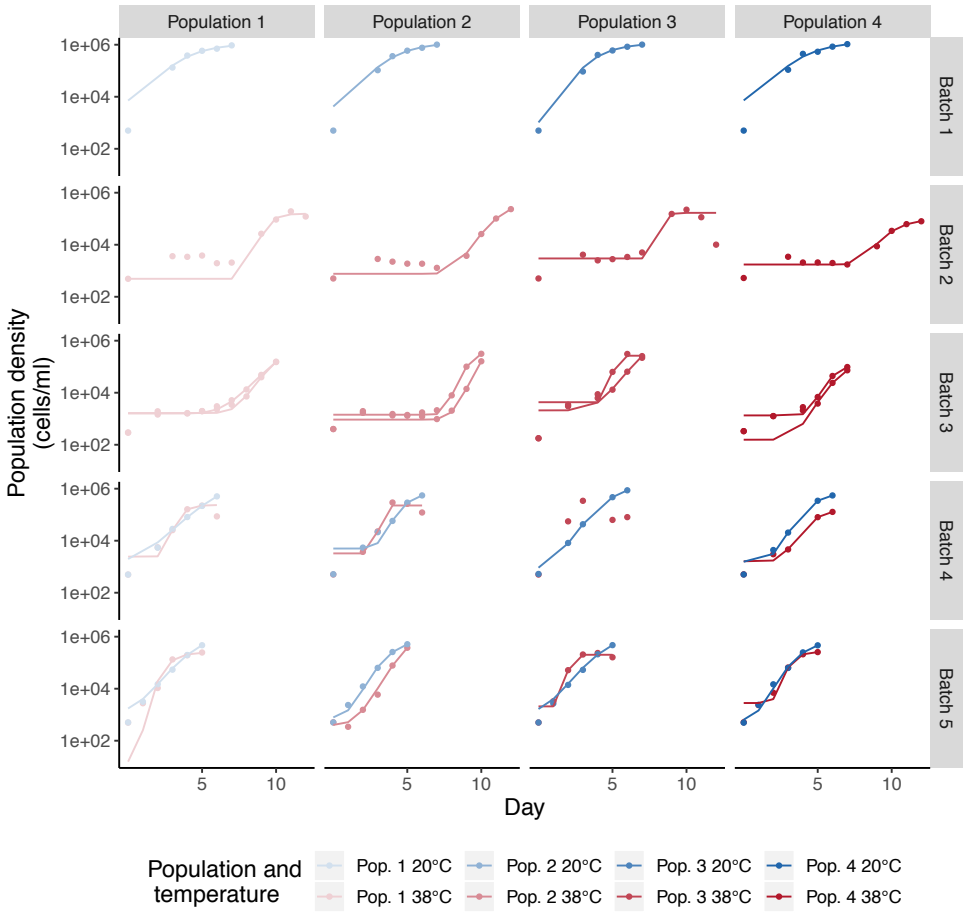

Figure S1 Population dynamics of *T. thermophila* analyzed with a Gompertz model. Points show population abundances and lines show the fitting of the Gompertz model. The colors indicate the temperature in which the population was grown, and the shades represent the population replicate. No model fit is shown for population 3 in batch 4 at 38 °C since growth parameters were manually calculated (see methods).

Chapter 3. Functional genomics of the ciliate *Tetrahymena thermophila* during temperature stress

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Abstract

Identifying the molecular mechanisms that allow species to adapt to novel temperatures can help us understand and predict species response to this key environmental factor. *Tetrahymena thermophila*, a unicellular eukaryote, is a model species in cellular biology and used in studies ranging from toxicology to community ecology. This species can grow in temperatures up to 40 °C, yet only a few molecular mechanisms that constitute its heat stress response have been studied. We exposed populations of *T. thermophila* to a novel high temperature environment for more than 35 generations and monitored cell functioning through transcriptome sequencing. Substantial transcriptional modulation in response to temperature change was observed. Repression of carbohydrate metabolism and proteolysis were important across the entire experiment, indicating energy balance has as a key role in survival at high temperatures. We also monitored transcriptome changes in the populations that returned to the original temperature after the adaptation to high temperature, and found evidence that transcriptional plasticity is an important mechanism in this species' response to temperature.

Keywords

Temperature adaptation, gene expression, plasticity, heat stress, *Tetrahymena thermophila*

1. Introduction

Temperature is one of the main abiotic factors affecting a wide range of biological processes: cell metabolism is greatly dependent on temperature, species have a thermal optimum in which growth is maximized, and biodiversity patterns at the landscape scale are determined by temperature (Hochachka and Somero 2002). Temperature can be a central driver of adaptation in natural populations, as previously described in ciliates (Gächter and Weisse 2006), fungal pathogens (Laine 2008), *Daphnia* (Yampolsky, Schaer, and Ebert 2013) and other species. Experimental evolution has also investigated temperature as a selective pressure, demonstrating its significant role in shaping adaptive processes of e.g. bacteria (Bennett and Lenski 2007; Saarinen et al. 2018), phytoplankton (Padfield et al. 2016), fungi (de Crecy et al. 2009) and yeast (e.g. Caspeta and Nielsen 2015; Huang et al. 2018).

High temperatures lead to a large number of alterations in the cell structure and functioning, including the formation of misfolded proteins, protein translation decrease, cytoskeleton damage and disruptions in the cell membrane (Richter, Haslbeck, and Buchner 2010). Previous studies have identified important mechanisms that allow cells to overcome these difficulties. Heat-shock proteins (Hsps) have a key role in the response to thermal stress by promoting the correct folding of nascent or misfolded proteins (Feder and Hofmann 1999; Richter, Haslbeck, and Buchner 2010). Protein degradation pathways are also activated upon heat stress to avoid the accumulation of these misfolded proteins, which can be toxic to the cells (Hilt and Wolf 1992; Meyer and Baker 2011). Modification of the lipid composition of the cell membrane, known as homeoviscous adaptation, is also an important mechanism to ensure membrane stability in a range of species (Guschina and Harwood 2006; Ernst, Ejsing, and Antonny 2016).

Tetrahymena thermophila is one of the best studied ciliate species, with important resources available, such as sequenced macro- and micro- nuclear genomes (Eisen et al. 2006; Hamilton et al. 2016) and gene expression profiling during its cell cycle (Miao et al. 2009; Xiong et al. 2012). This species can grow in a broad range of temperatures, with cell division possible at temperatures as high as 40 °C (Laakso, Löytynoja, and Kaitala 2003), making it an interesting eukaryote model system for temperature adaptation. A few mechanisms of the heat-shock

response have been studied in this species, such as the role of heat-shock proteins (Williams and Nelsen 1997; Ketola et al. 2004; Fukuda et al. 2015; Woehrer et al. 2015), however they mostly focused on short-term responses to temperature change. To date, general transcriptome profiling of this organism at high temperatures is absent, as well as during long-term heat stress.

In this study, we investigate the transcriptome response of *T. thermophila* exposed to a high temperature environment during more than 35 generations. We monitored transcriptome changes throughout the experiment, in order to identify short and long-term changes in cell functioning driven by temperature. We predict that the heat-shock proteins will be upregulated at high temperatures, though long-term expression of these proteins might be detrimental for the cells. An overall downregulation of the transcriptome is also expected, as cells at thermal stress reduce their energy consumption.

We also explored the stability of the temperature response by monitoring the transcriptome of populations that returned to the control temperature after many generations at 38 °C. Transcriptomes were assessed within the same generation and after many generations back at the control temperature, allowing us to identify transcriptionally plastic genes, since plasticity is a relevant mechanism for responses to environmental change (Schlichting and Smith 2002; Ghalambor et al. 2007).

2. Methods

2.1. Experimental design

The *T. thermophila* strain 1630/1U was used in this experiment. Individuals only reproduced clonally since they all present the same mating type. A detailed description of the experiment can be seen in Chapter 2 of this thesis and a schematic is shown in Figure 1. Two temperatures were used, 20 °C, which was the control condition and is a temperature in which this species reaches high growth rates, and 38 °C, which is close to the thermal limit of this species. The experiment comprised four separate evolving lineages in five subsequent batch cultures. Each lineage was initiated from a single stock culture, and in each batch they grew from low density (500 cells/ml) until carrying capacity was reached.

The experiment started with the four replicate populations growing at 20 °C (Figure 1). In batch 2, the four populations were exposed to 38 °C. In batch 3, eight populations were grown at 38 °C, since each population in the previous batch generated two new populations. At the end of batch 3, the cultures were exposed to either 20 or 38 °C for one hour, i.e. one population of each pair was moved to the control temperature of 20 °C, while the other population remained at 38 °C. In the subsequent batches, populations were maintained at the temperature they experienced during this one-hour exposure.

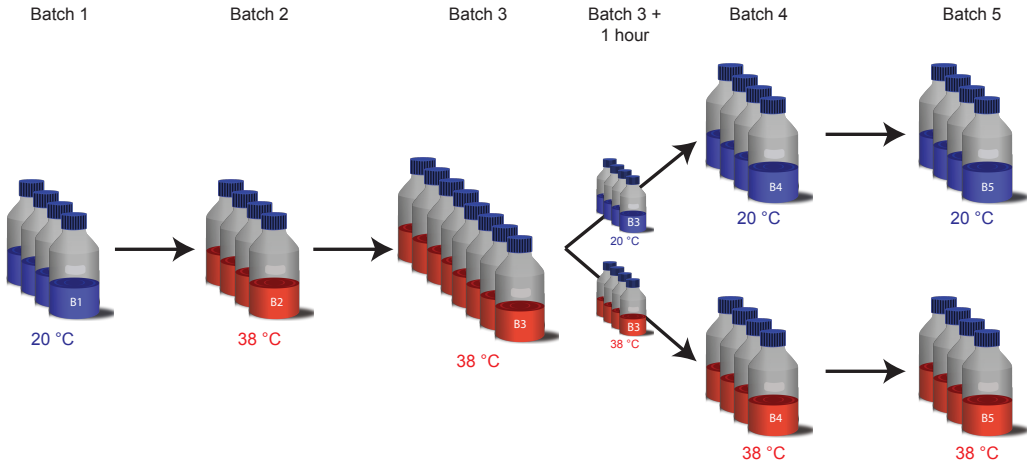

Figure 1 Experimental design of the temperature adaptation experiment. Each bottle represents one replicate batch culture, and the colors indicate the temperature at which the culture was grown: blue bottles at 20 °C, red bottles at 38 °C. Labels on top of the figure show sampling points for RNA-seq. Sampling occurred at the end of each batch, except in batch 3, in which populations were sampled one additional time, after half of the populations were moved to 20 °C during one hour, and the other half of the populations remained at 38 °C.

2.2. RNA-seq sampling and library preparation

Populations were regularly monitored by videography to estimate population abundances (see details of this method in Chapter 2). On the last day of each batch culture, during the stationary phase, populations were sampled for transcriptome analysis. One extra sampling was performed in batch 3, when populations were also

sampled after the one-hour temperature exposure (Figure 1), resulting in a total of 40 samples.

At each sampling point, we collected approximately 1.5 million cells from each population. Samples were centrifuged to separate the cells from the medium, and the pelleted cells were stored in RNAprotect cell reagent (Qiagen) at -80°C until further processing. This species presents nuclear dualism, i.e. two nuclei in each cell, but since only one nucleus is transcriptionally active, it is not necessary to separate them for gene expression analyses. Total RNA was isolated using the RNeasy mini kit (Qiagen) after cells were homogenized with Qiashredders (Qiagen). Quality control and estimation of RNA concentration was performed by Nanodrop and Qubit. Library preparation and sequencing of the samples was performed at Barts and The London Genome Centre at Queen Mary University of London. Libraries were sequenced on the Illumina NextSeq 500 platform, generating 125 bp paired-end reads.

2.3. RNA-seq data analysis

The quality of the RNA-seq reads was assessed with FastQC version 0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and MultiQC version 1.0 (Ewels et al. 2016), and low quality bases and adaptor contamination were removed with Trimmomatic version 0.38 (Bolger, Lohse, and Usadel 2014). The reads were aligned to the most recent version of the reference genome of *T. thermophila* (June 2014 version) available at the *Tetrahymena* Genome Database (Stover et al. 2012). Read mapping was performed with the aligner STAR version 2.6.1 (Dobin et al. 2013) using default parameters. A summary of the filtering and mapping rates of each sample can be found in Figure S1.

We performed differential expression analyses using Deseq2 version 1.24.0 (Love, Huber, and Anders 2014). We only included genes with a normalized read count larger than 10 in at least two samples. We included in the model population lineage, batch, temperature, and a variable describing if populations were sampled at the initial or late stationary phase. In summary, Deseq2 normalizes the RNA-seq counts for differences in sequencing depth between the samples and then fits a generalized linear model to each gene. The statistical significance of the parameters of the model is tested with a Wald test. Genes were considered differentially expressed when the $\log_2$ fold changes were larger than 2, ensuring our results reflect

large changes in gene expression. The Benjamini-Hochberg procedure was used to correct for multiple testing.

Two main differential expression analyses were performed. First, to identify the transcriptome response to high temperature, we compared the populations that were grown at 38 °C (batches 2 to 5) to the populations in batch 1 that were grown at 20 °C, identifying the downregulated and the upregulated genes at 38°C. The second analysis focused on the stability of the temperature response, comparing the paired populations in batches 3 to 5 that were grown at 38 °C versus the ones that returned to 20 °C.

To identify the functions of the genes that were differentially expressed, gene ontology (GO) enrichment analyses were performed with GOseq version 1.36.0 (Young et al. 2010), using GO categories that included a minimum of ten genes. GO terms can be part of three different domains, molecular function, cellular component and biological process; our analyses included all three domains. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed with clusterProfiler version 3.12.0 (Yu et al. 2012).

3. Results

3.1. Gene expression changes in response to temperature stress in *T. thermophila*

Principal component analysis of all genes in the 40 transcriptome samples revealed three main clusters, one for each temperature and a third separate cluster which comprised samples from both temperatures (Figure 2). Experimental design, batch variation during sequencing and the quality of the sequencing data did not explain the differences between these three clusters. The only factor that appeared related to these differences was the growth stage at which transcriptome sampling occurred. Even though all populations were sampled at the stationary phase, most of the populations in this third cluster were sampled at the late stationary phase, after population density started to decline. Population growth dynamics has an impact on the gene expression patterns of *T. thermophila* (see Chapter 4 of this thesis) and we therefore included this factor when modeling gene expression changes.

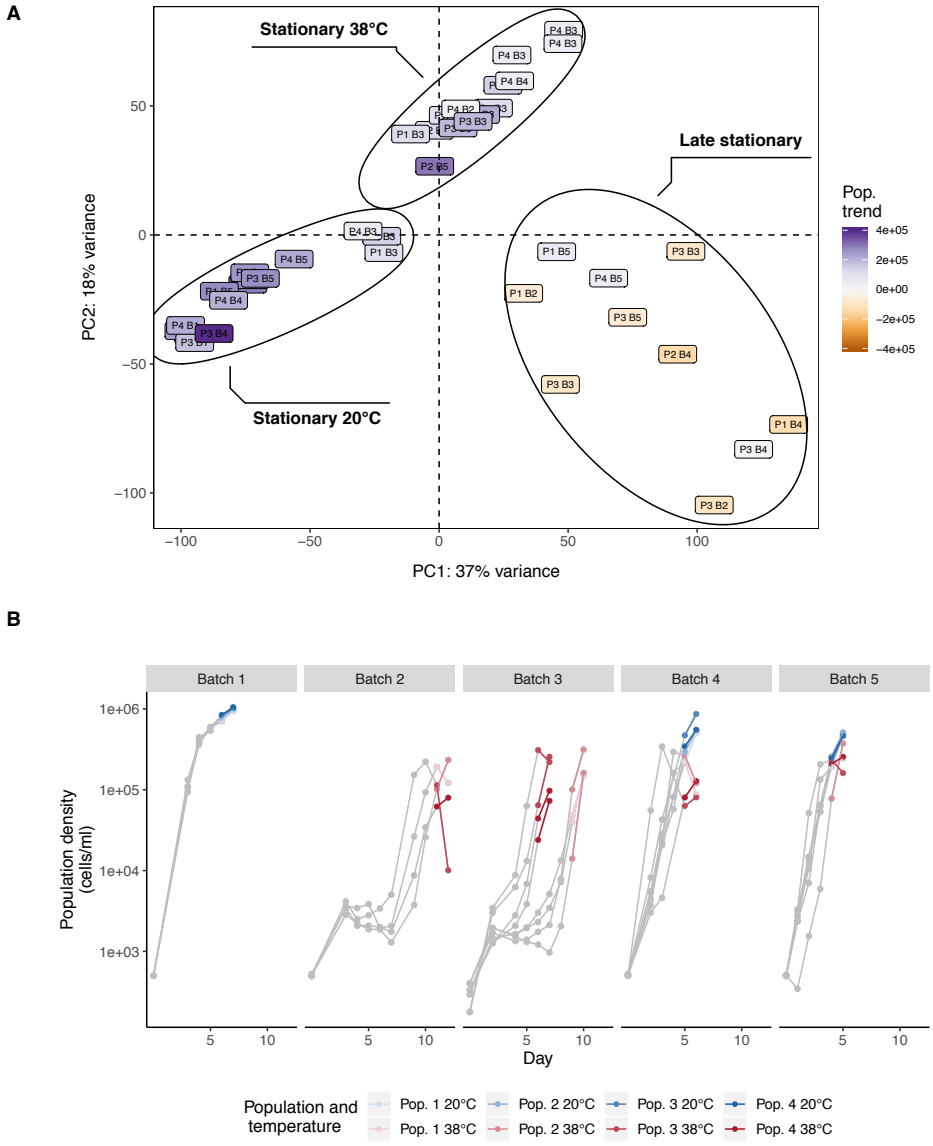

Figure 2 Detection of growth phase effects in the transcriptome data. A) Principal component analyses of all the RNA-seq samples based on regularized log counts. The name of each sample represents the population lineage (P1 to P4) and the experimental batch (B1 to B5). The samples are colored according to the population trend when sampling occurred; purple indicates population density was still increasing, while orange indicates population density was decreasing. B) Population dynamics of *T. thermophila* during the experiment. Populations were sampled for transcriptome analyses at the last day of each batch. The last two measured population abundances (colored) were used to calculate the population trend in plot A. The colors indicate the population lineage and the temperature in which the population was grown.

Exposure to high temperature led to extensive changes in the transcriptome of this species. We found 6057 differentially expressed genes (DEG) throughout the

experiment (Figure 3A). We identified a core gene expression response of 1762 genes (29 % of the DEG in the experiment) that were differentially expressed in all four batches (Figure 3B), indicating that the expression of many of the genes was only transient during the experiment.

Batch 3 presented the largest number of DEG and also the largest proportion of batch-specific DEGs, with 27% of the DEG uniquely expressed at this time point (Figure 3B). This batch is composed of eight replicate populations at the same temperature, while all the other batches have only four populations per temperature. To check if this larger sample size could lead to a higher number of detected DEG, we reanalyzed the data including only four randomly chosen populations from batch 3. The same pattern was observed even when sample size was equal across all batches: batch 3 presented the largest number of DEG in all analyses (not shown). We therefore believe this pattern is not driven by a larger sample size and report the results of the differential expression analyses for the full dataset.

Functional enrichment analyses revealed 13 GO terms overrepresented in the upregulated genes at 38 °C, and many of these categories were specific to one or two batches (Figure 4A). A different pattern was found among the down-regulated genes, with five of the six overrepresented GO terms present in all batches. Downregulated categories related to proteolysis and carbohydrate metabolism were observed throughout the entire experiment. The upregulated terms included ATPase activity and the cell compartment membrane, which were enriched in three batches, and other functions related to ion transport, chromatin silencing, protein modifications and response to oxidative stress.

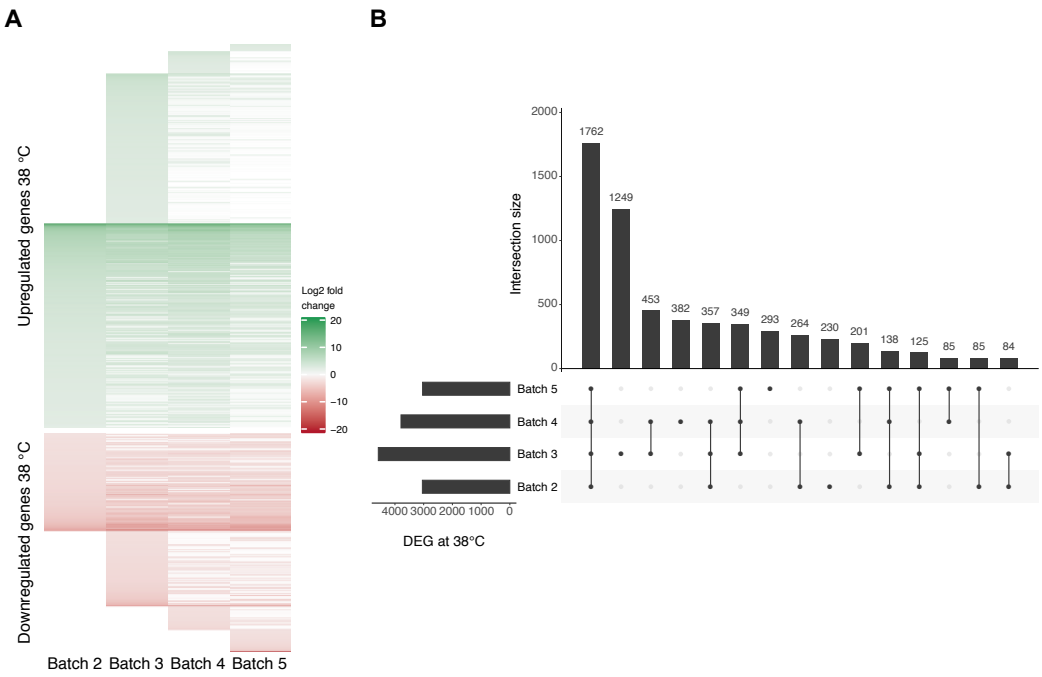

Figure 3 Transcriptome response to high temperature in *T. thermophila*. (A) Heatmap of the estimated log_2 fold changes in gene expression at 38 °C. Each row represents one gene, and each column represents the batch used to estimate gene expression changes (see experimental design in Figure 1). The color of each cell represents the estimated log_2 fold change, and white cells are non-significant changes. Transcriptome of populations at 20 °C in batch 1 was used as reference. Adaptive shrinkage estimator (Stephens 2017) was used to shrink the log_2 fold changes for better visualization. (B) UpSet plot of the intersection between the sets of differentially expressed genes (DEG) in each batch. The vertical bar plot represents the intersection size, with the number of genes in each group on top. The dot plot shows the batches present in each intersection, and the horizontal bar plot reports the number of DEG in each batch.

We found six KEGG pathways overrepresented in the upregulated genes, with only the pathway SNARE (soluble NSF attachment protein receptors) interactions in vesicular transport present in all four batches (Figure 4B). We observed ten downregulated pathways at 38 °C, with two pathways, sphingolipid metabolism and folate biosynthesis, enriched in all batches. Similarly to the GO categories, many KEGG pathways were downregulated in most batches, while the activated pathways presented batch-specific patterns.

To further explore the gene expression changes observed in this experiment, we focused on a few genes that were previously related to heat stress or other

environmental stressors in *T. thermophila*. Heat-shock proteins are part of the thermal stress response in all organisms and, as expected, we found eight HSP genes upregulated at 38 °C, even though only three of them were upregulated during the entire experiment: *SSA3*, *Hsp71* and the gene *TTHERM_00895620*, a putative DnaK protein (Figure S2). All three genes belong to the Hsp70 family and previous studies have found an upregulation of Hsp70 genes when cells go through starvation (Fukuda et al. 2015), indicating they respond to a range of stressors, as is commonly seen in these proteins. HSP have putative high costs to the cells (Feder and Hofmann 1999), and this long-term upregulation might be related to the longer lag phases or smaller cell sizes observed at 38 °C.

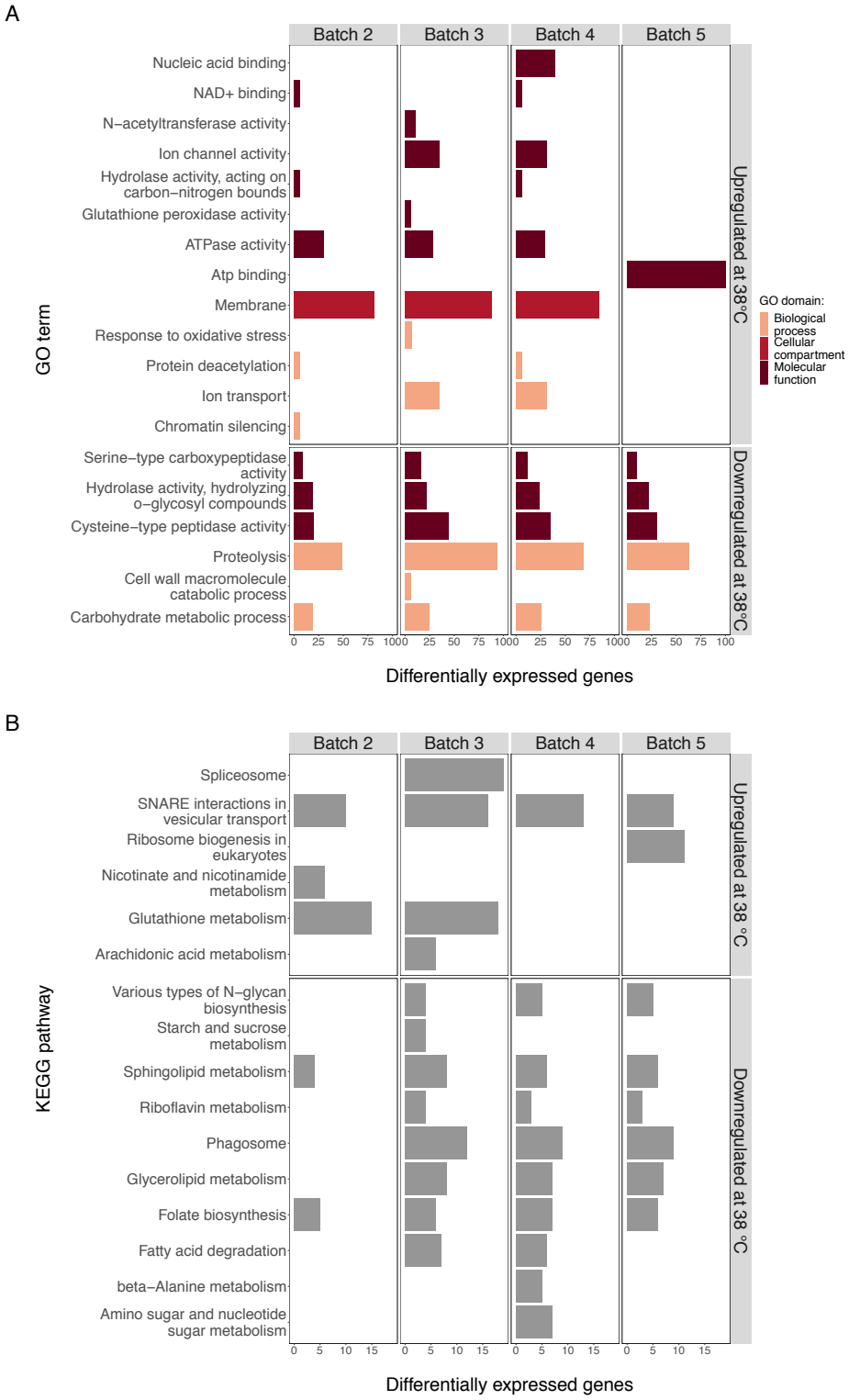

Figure 4 Functional enrichment analyses of the differentially expressed genes at 38 °C in *T. thermophila*. Plots show the overrepresented gene ontology terms (A) and KEGG pathways (B) in the downregulated and upregulated genes in each batch (adjusted *p*-value < 0.05). Y-axes show the name of each GO term or pathway, and x-axes show the percentage of genes in each

category that were differentially expressed. GO terms are divided into three domains, which are represented by the different colors.

Metallothioneins (MT) form a family of proteins mainly responsible for detoxification of heavy metals through bioaccumulation (Juan C. Gutiérrez, Amaro, and Martín-González 2009; Juan Carlos Gutiérrez et al. 2011). However, their expression can be stimulated by a wide range of environmental stressors beyond metals, including temperature shocks, pH, and starvation (Dondero et al. 2004; Díaz et al. 2007; Juan Carlos Gutiérrez et al. 2011). *T. thermophila* has five MT genes and all of them were differentially expressed at high temperature in this study (Figure S3). Four genes were upregulated while one was downregulated at 38 °C. Two genes, *MTT2* (a copper-MT) and *MTT3* (a cadmium-MT), were upregulated in all four batches. These results are in accordance to the hypothesized role of these proteins not only on heavy metal detoxification, but on general cell protection again stress, which is probably related to a strong antioxidant activity (Dondero et al. 2004).

3.2. Stability of the transcriptome response

To explore the stability of the temperature response, we analyzed the transcriptome changes that occurred when the populations returned to 20 °C after two batches growing at 38 °C. These analyses can be divided into two categories, the within generation response, which occurred in the one-hour temperature change in batch 3, and the intergenerational response, comprising batches 4 and 5 (see experimental design in Figure 1).

An overview of the changes in the transcriptome can be seen in the principal component analysis in Figure 5. The one-hour exposure to 20 °C strongly affected the transcriptomes of the populations, as can be seen by the large distance between the samples before and after the one-hour test (samples connected by lines in Figure 5). After one hour at 20 °C, all four populations moved in the direction of the populations in batch 1 at 20 °C, used as a reference in this experiment, displaying a very consistent effect among all populations. The intergenerational effect was stronger, and populations clustered even more closely to the reference populations in batch 1, even though a larger diversity was seen between the population replicates in batches 4 and 5, compared to the control in batch 1.

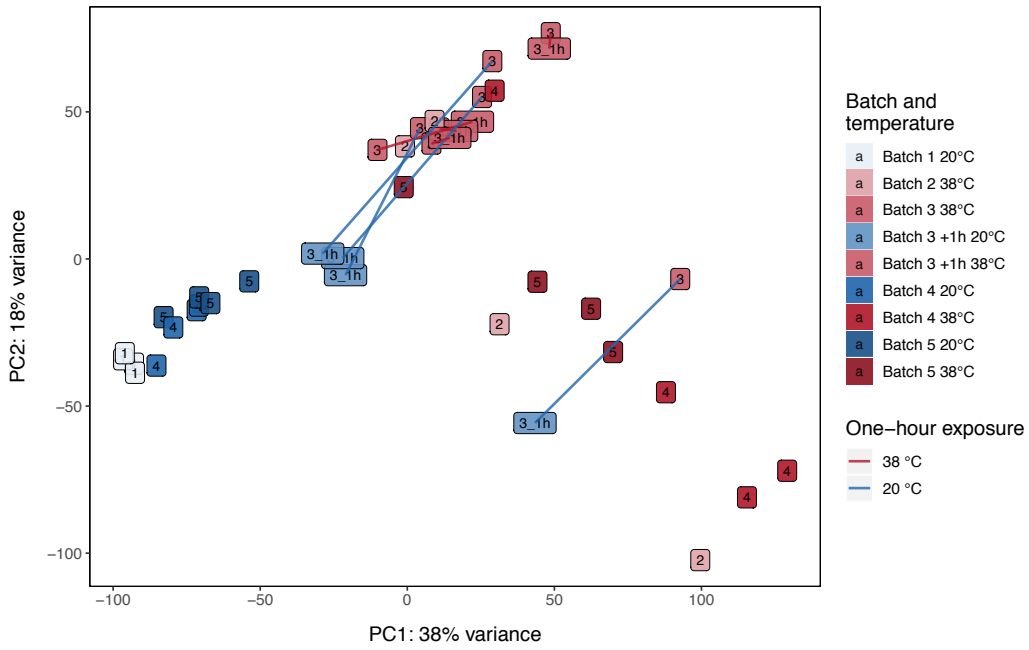

Figure 5 Principal component analysis of the transcriptomes of *T. thermophila* during temperature adaptation. Analysis is based on the RNA-seq counts after regularized log transformation. Samples are named according to the batch in which they were sampled. A line links the same populations before and after the one-hour exposure. The colors mark the temperature in which the populations were grown, and shades represent the different batches.

To further detail the transcriptome changes that occurred when populations returned to 20 °C, we performed differential expression analyses using the populations at 38 °C in the same batch as a reference. We observed 4901 genes that were differentially expressed in these comparisons (Figure 6A) and most of the genes were uniquely expressed at the individual time points (Figure 6B). This pattern is in contrast to what we observed in the transcriptome response to 38 °C, in which many genes were differentially expressed across all batches (Figure 3B).

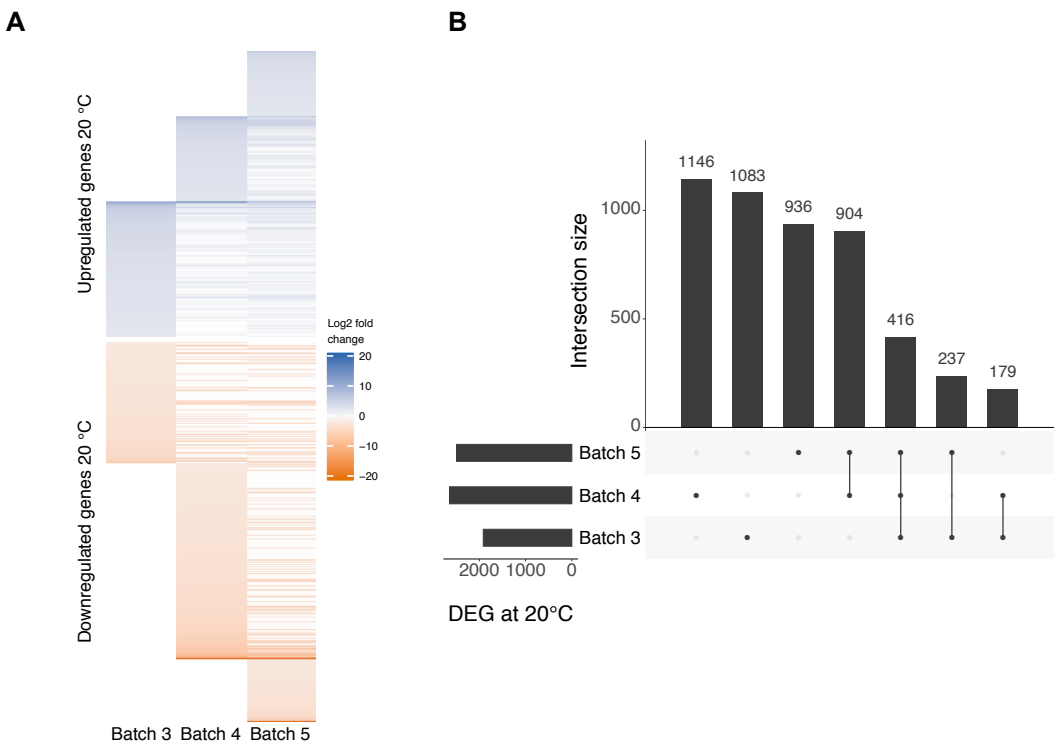

Figure 6 *T. thermophila* transcriptome changes at 20 °C after 17 generations at high temperature. A) Heatmap of the estimated log_2 fold changes in gene expression at 20 °C. Each row represents one gene, and each column represents the batch used to estimate gene expression changes. The color of each cell represents the estimated log_2 fold change, and white cells are non-significant changes. Transcriptome of populations at 38 °C in the respective batches were used as reference. Adaptive shrinkage estimator (Stephens 2017) was used to shrink the log_2 fold changes for better visualization. B) UpSet plot of the intersection between the sets of differentially expressed genes (DEG) in each batch. The vertical bar plot represents the intersection size, with the number of genes in each group on top. The dot plot shows the batches present in each intersection, and the horizontal bar plot reports the number of DEG in each batch.

An overview of the results of the functional enrichment analyses is shown in Figure 7. Nine GO terms were enriched in the upregulated genes at 20 °C, while ten GO terms were enriched in the downregulated genes (Figure 7A). The KEGG enrichment analyses found 13 pathways enriched in the upregulated genes, and eight pathways enriched in the downregulated genes (Figure 7B). In both analyses, most categories were unique to one of the batches, further evidence of the specificity of the genes modulated at each time point. Many of the downregulated cell functions at 38 °C (Figure 4) were upregulated when populations returned to 20 °C (Figure 7).

Proteolysis for example, was immediately upregulated after one hour at 20 °C, while carbohydrate metabolism was upregulated later, in batches 4 and 5.

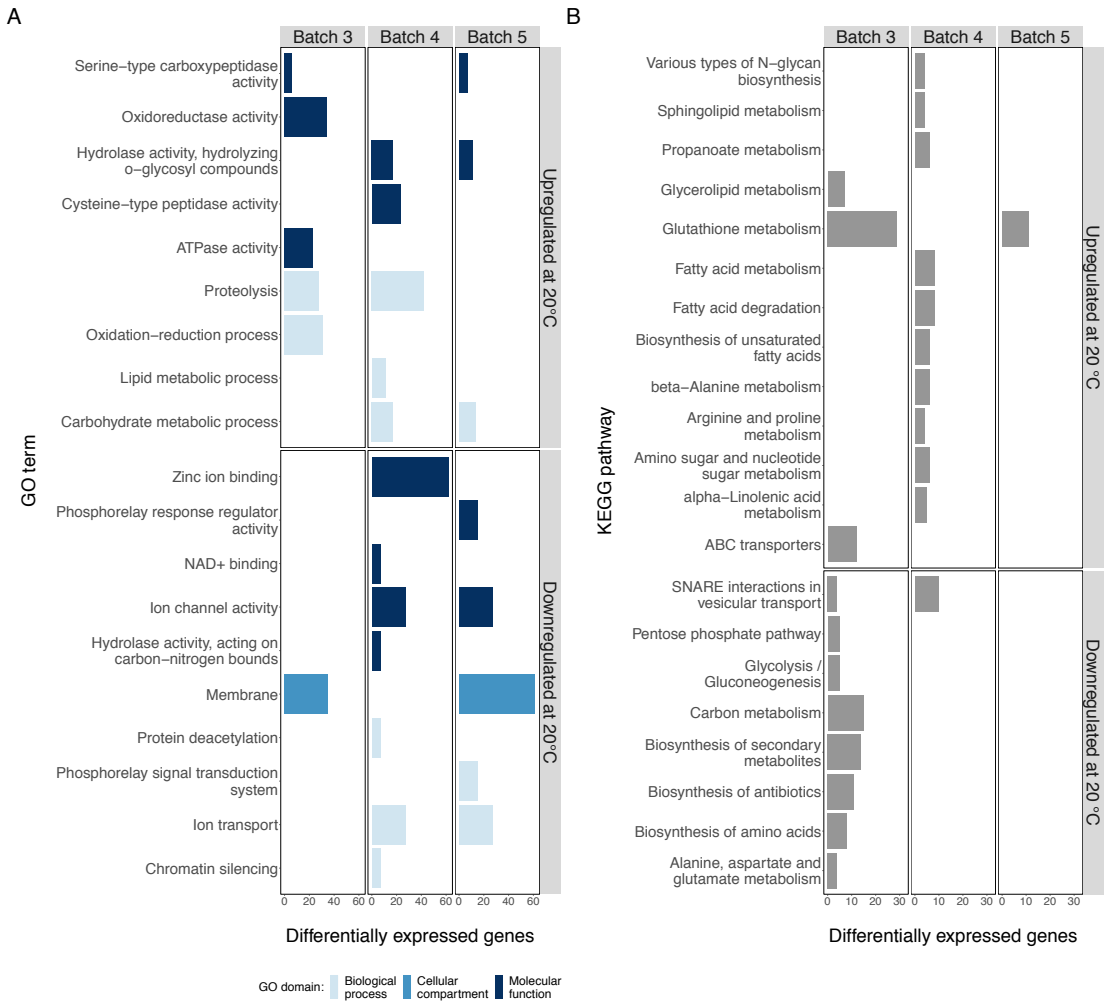

Figure 7 Functional enrichment analyses of the differentially expressed genes at 38 °C in *T. thermophila*. Plots show the overrepresented gene ontology terms (A) and KEGG pathways (B) in the upregulated and downregulated genes in each batch (adjusted *p*-value < 0.05). Y-axes show the name of each GO term or KEGG pathway, and x-axes show the number of genes in each category that were differentially expressed. GO terms are divided into three domains, which are represented by the different colors.

4. Discussion

4.1. General response to high temperature in *T. thermophila*

Transcriptome profiling of the ciliate *T. thermophila* exposed to high temperature revealed extensive gene expression changes. The core temperature response, composed of genes differentially expressed during more than 35 generations, includes 1762 genes, which represent 6.5 % of this species' transcriptome. Different mechanisms can induce a heat stress response, such as transcriptional modulation (e.g., Gasch et al. 2000; Sørensen et al. 2005; Gunasekera, Csonka, and Paliy 2008), translational modulation (Shalgi et al. 2013) and also post-translational protein modifications (e.g., Chhabra et al. 2006; Seifert et al. 2015). Our results indicate that transcriptional regulation is a key component of the heat stress response in this species. Chromatin silencing, for example, an important transcriptional modulator, was an upregulated function in the first batch of the experiment (Figure 4A).

High temperature environments are known to lead to oxidative stress and protein damage, and we indeed observed molecular functions such as glutathione peroxidase activity and biological process such as response to oxidative stress upregulated at 38 °C. Interestingly, these functions were mostly enriched in the initial batches, when populations still presented long lag phases (Chapter 2). We also observed the upregulation of proteins known to be important to the stress response in *T. thermophila* and other organisms, such as the heat-shock proteins and the metallothioneins. Some of these genes were differentially expressed in specific batches, while others were upregulated during the entire experiment, probably reflecting different roles in the short or long term response to temperature.

Previous studies in aquatic organisms, including *Daphnia* and corals, have observed a larger number of genes downregulated at high temperatures, in comparison to the number of upregulated genes (Yampolsky et al. 2014; Levin et al. 2016), a pattern that has been interpreted as a general downregulation of the cell functions in order to conserve energy. In our study, a more complex pattern was found. We observed an excess of upregulated genes at 38 °C, in comparison to the downregulated genes (Figure 3), suggesting that global downregulation of gene expression is not part of the thermal stress response. However, most of the enriched

functional categories among the downregulated genes at 38 °C were related to metabolism, including carbohydrate metabolism (Figure 4), so decrease in energy consumption and metabolism repression also seem to be important in *T. thermophila*.

The most consistent pattern of functional enrichment was found among the downregulated genes, with proteolysis and carbohydrate metabolism enriched in all four batches (Figure 4). Proteolysis is usually a cell function upregulated in response to high temperatures, since it is important for the degradation of misfolded proteins. The observed downregulation in *T. thermophila* might indicate that other mechanisms respond to the increase in misfolded proteins, such as the activation of chaperones. Proteolysis might also be downregulated at high temperatures because the production of proteases is linked to nutrient uptake in *T. thermophila*, since this species excretes proteases into the external environment (Herrmann et al. 2006; Madinger et al. 2010). This would indicate that downregulation of proteolysis is also related to the repression of cell metabolism.

We found significant pathways and biological processes enriched in the differentially expressed genes of *T. thermophila* exposed to high temperature, as discussed above. Importantly, the genome annotation of this species is still largely incomplete, with only 23 % of its genes annotated with a GO term and 7 % with a KEGG pathway. Among the 6057 differentially expressed genes at 38 °C, 2112 genes (34.5 %) are annotated as hypothetical proteins, with no other information about possible molecular function. Consequently, only a reduced subset of the differentially expressed genes could be explored with the functional enrichment analyses, and many important biological processes might not be identified. This is a common issue among many species and highlights the need for better genome annotations and functional characterization of proteins (Galperin and Koonin 2010).

4.2. Stability of the temperature response

After around 20 generations in a high temperature environment, the populations of *T. thermophila* were returned to the original temperature of 20 °C. We first monitored the transcriptome changes that occurred within one hour in order to identify the genes with a plastic response, since cell division should be limited in that amount of time. A large number of genes were differentially expressed within this period, indicating a plastic response; upregulated plastic genes were mostly related

to proteolysis and oxidation-reduction processes, while the downregulated genes were responsible for the metabolism of different amino acids (Figure 7).

Plasticity can be beneficial for species that face changing environments (Stern et al. 2007; Gienapp et al. 2008) and there is evidence that enhanced plasticity can be selected as a response to abiotic stress. It is therefore not surprising that many genes displayed a plastic response in our experiment. It is important to emphasize that one hour might not be enough time for all cell functions to respond to the temperature change and a larger number of genes might also be plastic, even though they were not detected in our study.

At the end of batch 4, populations have experienced roughly 10 generations back at 20 °C and their transcriptomes are very similar to the ones observed in the ancestral populations also at 20 °C (Figure 5). This indicates that cells can quickly reestablish cell functioning at their original environment, despite 18 generations growing at a high temperature. Plastic genes might also be related to this fast return to normal cell functioning.

4.3. Conclusions

We explored the transcriptome response of the ciliate *T. thermophila* exposed to 38 °C during more than 35 generations. Repression of proteases and carbohydrate metabolism are key to survival at high temperature in this species, while a variety of cell functions are upregulated during the adaptive process. We see the upregulation of genes know to act in the heat-shock response, but also of genes related to other abiotic stressors, indicating the transcriptional response to stress has many components that respond to a large variety of environmental changes. Many genes displayed transcriptionally plastic patterns, pointing to the relevance of this mechanism in the temperature response. A better annotation of the genome of *T. thermophila* might identify additional components of its temperature response. Furthermore, functional experiments such as gene knockdowns would be interesting to test the relevance of the identified cell functions and pathways and to evaluate their possible fitness consequences.

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Supplementary material

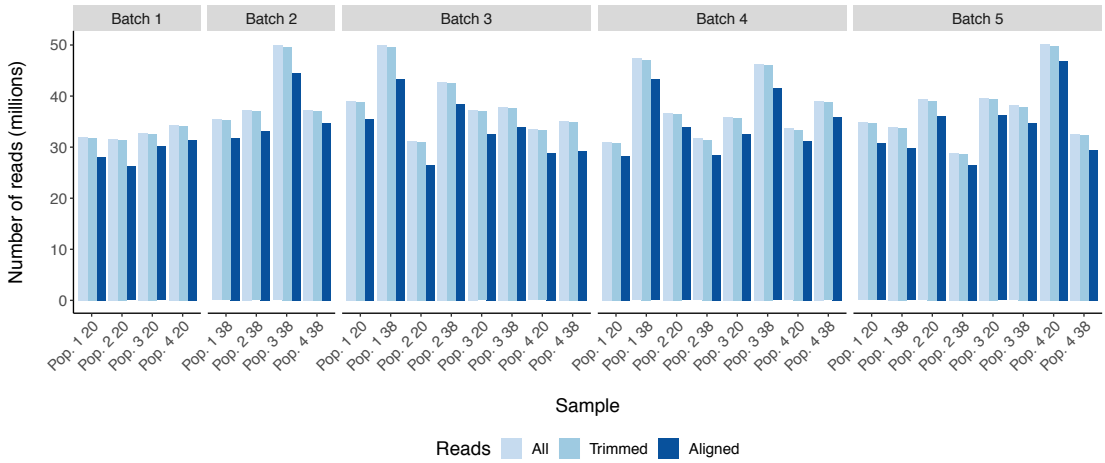

Figure S1 Summary of the quality control and alignment of the RNA-seq reads. Each group of three bars represents one sample, and the sample name is composed of the number identifying the experimental population followed by the temperature in which it was grown. The samples are grouped according to the batch culture in which they were grown. The bars show total number of reads obtained in the sequencing, the reads that survived quality filtering and the reads that were uniquely aligned to the reference genome (Q>30).

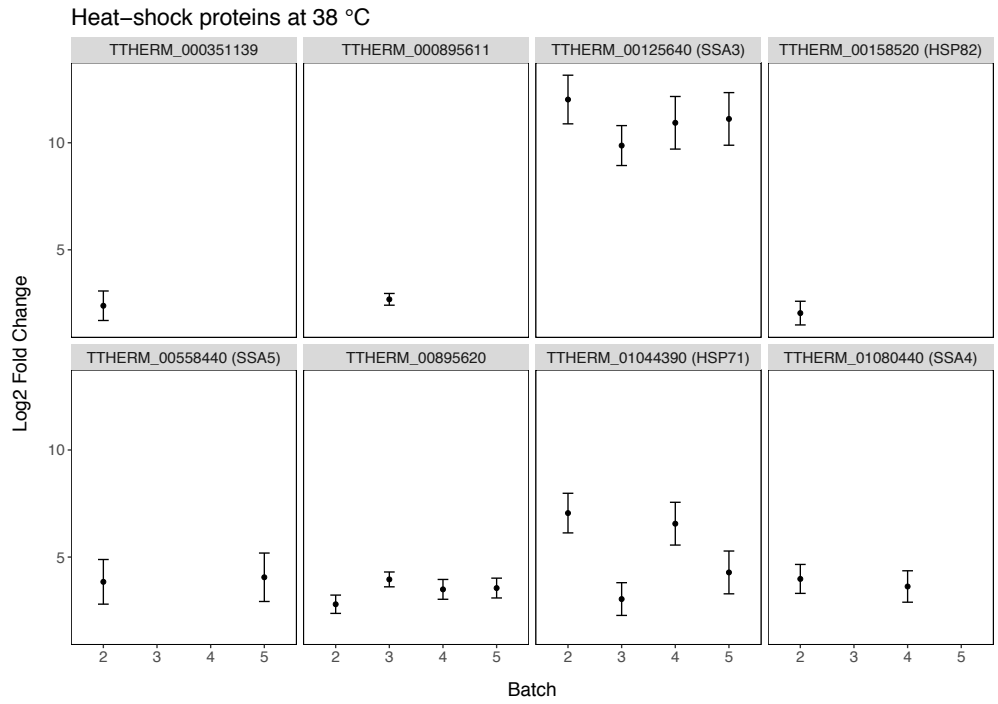

Figure S2 Heat-shock proteins differentially expressed at 38 °C in *T. thermophila.* Each plot shows the estimated log_2 fold change (LFC) in gene expression and error bars represent the standard error. Only genes with LFC > 0 are shown. Populations at 20 °C in batch 1 are used as reference. Gene code is shown on the top of each plot, with the name of the protein between parentheses, when available.

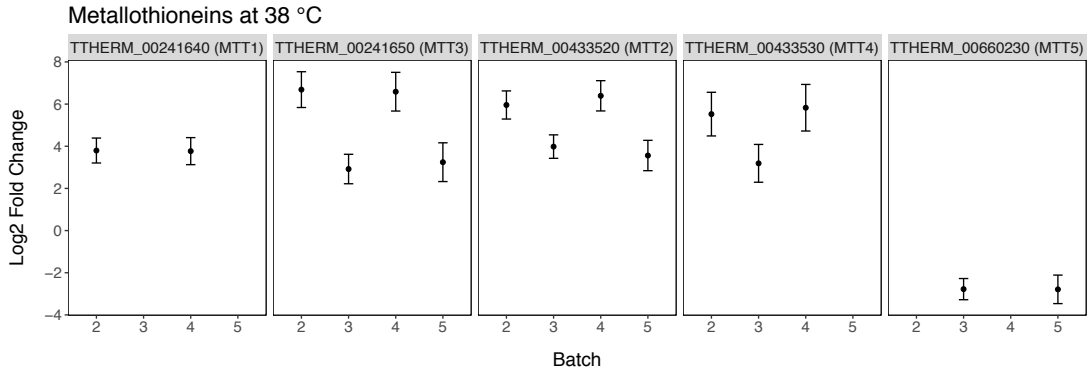

Figure S3 Metallothioneins differentially expressed at 38 °C in *T. thermophila.* Each plot shows the estimated log_2 fold changes in gene expression and error bars represent the standard error. Populations at 20 °C in batch 1 are used as reference. Gene code is shown on the top of each plot, with the protein name between parentheses.

Chapter 4. Gene expression patterns and the role of epigenetics during growth of the ciliate *Tetrahymena thermophila*

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Abstract

Population growth and its density dependence is a key biological process with implications for ecological and evolutionary dynamics. Using the unicellular eukaryote *Tetrahymena thermophila*, we explored morphological traits and gene expression dynamics during a period of logistic-type growth. While small changes were observed at the phenotypic level, large differences in gene expression between the rapid growth phase and the stationary phase were detected, with nutrient availability and energy balance probably playing important roles in these dynamics. We also investigated the role of two histone post-translational modifications (PTMs), H3K4me3 and H3K9ac, in the observed changes in gene expression. Both histone marks had similar genome-wide distributions, with an enrichment downstream of the transcription start sites, marking expressed genes in this species. Most of these regions displayed stable distribution of histone PTMs throughout logistic growth, indicating that the gene expression changes are probably modulated by different histone PTMS or other gene expression modulators.

Keywords

Population growth, transcriptome, histone post-translational modification, growth phases, *Tetrahymena*

1. Introduction

The fate of a population can be highly dependent on its density. Too few individuals, and reproduction is reduced by difficulties in finding suitable mates; too many individuals and competition for resources also reduces reproductive rate. This is an example of a density-dependent process, i.e. one that is affected by changes in population density. A large number of biological processes display density-dependent dynamics. Competition between the individuals of the same species, predator-prey interactions and dispersal are examples of density-dependent ecological processes (Antonovics and Levin 1980; Boyce 1984). Evolutionary processes can also be affected by population density, such as density-dependent selection (Mueller 1997).

The dynamics of the previously described population can be termed as logistic growth, one of the models used to understand the density dependence of population growth. Logistic growth is characterized by a lag phase with slow growth, a sharp increase in population density during the rapid growth phase followed by a stable population density in the stationary phase. Natural populations show this type of growth, and it is also used to model laboratory populations that grow with limited resources such as space and nutrients.

In this study, we investigate the molecular basis of population growth and density dependence using the ciliate *Tetrahymena thermophila*. Different aspects of population growth have been studied in *Tetrahymena*, such as the DNA/histone ratio during growth (Stone 1969) or the effect of temperature on growth rate (Frankel and Nelsen 2001), and growth assays are frequently used in toxicological studies with this species (Schultz 1997; Gao et al. 2013).

Previous investigation by Miao et al. and Xiong et al. (2009; 2012) examined three different life cycle stages of this species: growth, conjugation and starvation. Using different molecular methods, they found strong differences between the gene expression patterns of these three stages. We believe the growth phase can be further explored at a more detailed scale, so here we analyze the transcriptome of *T. thermophila* populations at six different time points during logistic growth dynamics, covering the beginning of the rapid growth phase until late stationary phase.

Besides monitoring gene expression, we additionally assess the genome-wide distribution of two histone post-translational modifications (PTMs), trimethylation of histone H3 at lysine 4 (H3K4me3) and acetylation of histone H3 at lysine 9

(H3K9ac). In this species, the transcriptionally active macronuclei are enriched with H3K4me3 (Strahl et al. 1999) and also with different acetylated histones (Vavra, Allis, and Gorovsky 1982; Johmann and Gorovsky 1976), and these histone PTMs are associated with active transcription in other species (reviewed in Li, Carey, and Workman 2007). We expect to identify the main genes acting in each period of the logistic growth, and also to clarify the role of these two histone PTMs in the observed gene expression patterns.

2. Material and methods

2.1. Experimental design

The ciliate *Tetrahymena thermophila* (Figure 1A) strain 1630/1U was acquired from the Culture Collection of Algae and Protozoa. All cultures were grown in axenic conditions in 2 % proteose peptone medium. The stock culture was maintained at 15 °C, a temperature in which cultures can be cultivated over long periods. The experiment was performed in two identical blocks that lasted 9 days. In each block, 16 populations of *T. thermophila* were cultured in 2-liter bottles, each containing 500 ml of medium. Cultures were kept in incubators at 30 °C, a temperature that allows fast and stable growth of this species, and on shakers at 150 rpm to increase culture aeration. All populations in each block were started from a single stock culture at the initial density of 500 cells/ml.

2.2. Population abundances and cell morphology

We daily monitored all populations for estimating population density and for gathering morphological data on the cells. We sampled each population twice, removing 1000 µl of culture, since two replicates increase the accuracy of population density estimation. A five second video at 25 frames per second was taken of each sample, using a stereomicroscope (Leica M205 C) mounted with a digital CMOS camera (Hamamatsu Orca C11440, Hamamatsu Photonics, Japan). After the third day of the experiment, when population density was above 20,000 cells/ml, samples were diluted with sterile medium before the videos. We analyzed the videos with the

BEMOVI package (Pennekamp, Schtickzelle, and Petchey 2015) in R, identifying the number of cells in each video and measuring the cell area and the cell shape of all the individuals.

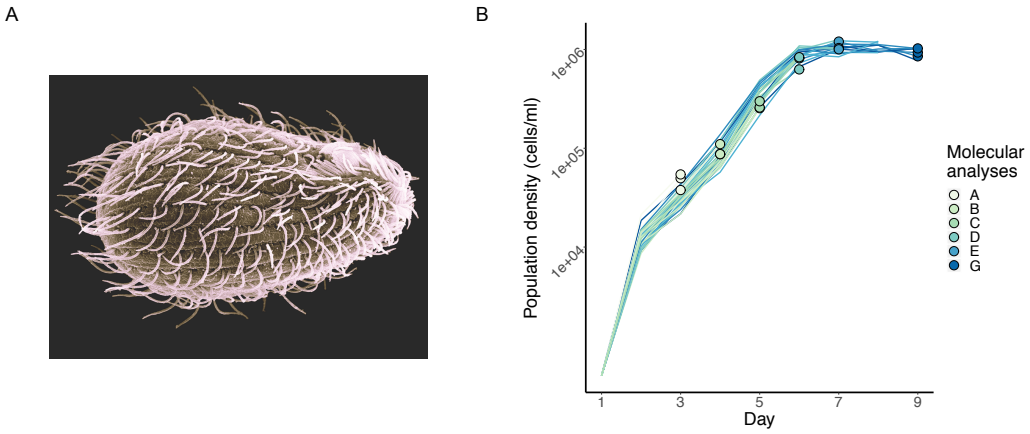

Figure 1 Picture of the ciliate *Tetrahymena thermophila* (A) and population dynamics during growth (B). Each line represents one of the 32 populations, and the circles represent the six sampling points for the molecular analyses (transcriptome and histone PTMs). The color of the lines indicates which populations were sampled for the molecular analyses at the same time. Picture credit: Dr. Aaron J. Bell

2.3. RNA isolation and RNA-seq library preparation

We destructively sampled the experimental populations for RNA-seq and ChIP-seq analyses in six different time points, starting on the third day of the experiment, when populations are in the rapid growth phase, until day nine, when populations are at the stationary phase (Figure 1B). Since a large number of cells were removed, leading to strong changes in the population dynamics, the populations were not grown after sampling for molecular analyses.

In each sampling point, three replicate populations were sampled. We removed 1.5 million cells of each population and stored them in RNAprotect cell reagent (Qiagen) at -80 °C. Cells were homogenized with Qiashredders (Qiagen) and RNA was isolated with RNeasy mini kit (Qiagen). Quality and concentration of RNA samples were estimated with Nanodrop and Qubit. Library preparations and sequencing were performed by the Functional Genomics Center Zurich, using

TruSeq Stranded mRNA kit (Illumina) with polyA enrichment and sequencing the libraries on an Illumina NovaSeq 6000, generating 101 bp single-end reads.

2.4. RNA-seq analysis

After checking the quality of the reads with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and MultiQC (Ewels et al. 2016), we trimmed low quality bases and removed adaptor contamination with Trimmomatic (Bolger, Lohse, and Usadel 2014). Using STAR (Dobin et al. 2013), we mapped the reads to the reference genome of *T. thermophila* (June 2014 version) obtained from the Tetrahymena Genome Database (Stover et al. 2012). A summary of the quality control and mapping rates of each sample can be seen in Figure S1.

We used DESeq2 (Love, Huber, and Anders 2014) for differential expression analyses, performing pairwise comparisons between all six sampling points. Genes were considered differentially expressed if the $\log_2$ fold change in the estimated gene expression was larger than 2, and *p*-values were corrected for multiple testing with Benjamini-Hochberg procedure (adjusted *p*-value <0.01). Gene ontology (GO) enrichment analyses were performed with GOseq (Young et al. 2010) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed with clusterProfiler (Yu et al. 2012).

2.5. Chromatin immunoprecipitation (ChIP) and ChIP-seq library preparation

All populations that were sampled for RNA-seq analysis were also sampled for ChIP-seq analysis at the same time. We sampled 20 million cells of each population and immediately performed nuclei isolation using a modified protocol based on the method of Sweet and Allis (2006) in which a glass dounce was used to disrupt the cells. Isolated macronuclei were cross-linked with 1% formaldehyde for 5 minutes and stored in dry pellets at -80°C. Chromatin shearing was performed in a M220 Focused-ultrasonicator (Covaris) for 20 minutes. Immunoprecipitation was performed using the iDeal ChIP-seq kit for Histones (Diagenode) and the following antibodies: H3K4me3 (Abcam, ab8580, 0.9 µg per ChIP) and H3K9ac (Active Motif,

39137, 1 µl per ChIP). A control immunoprecipitation without any antibody was performed in parallel. DNA was purified with MinElute PCR purification kit (Qiagen).

Library preparations and sequencing of immunoprecipitated DNA and the control were performed by the Functional Genomics Center Zurich. Libraries were performed with NEBNext Ultra DNA Library Prep kit (New England Biolabs) and samples were sequenced on an Illumina HiSeq 2500, resulting in 126 bp single-end reads.

2.6. ChIP-seq analyses

We trimmed reads with low quality bases using Trimmomatic (Bolger, Lohse, and Usadel 2014) and we used Bowtie2 with default parameters (Langmead and Salzberg 2012) to align the reads to the reference genome of *T. thermophila*. A summary of the quality control and mapping of each sample can be found in Figure S2.

MACS2 (Zhang et al. 2008) was used to identify the binding regions (peaks) of the histone PTMs, using the narrow peak method. DiffBind (Stark and Brown 2011) was used to find a set of common peaks between the samples, selecting peaks that were present in at least two replicates within each sampling point. Differential enrichment analysis was performed with the EdgeR method (Robinson, McCarthy, and Smyth 2010), and ChIPseeker (Yu, Wang, and He 2015) was used to annotate the peaks to the closest gene.

3. Results

3.1. Cell morphology during logistic growth

Populations displayed very similar growth dynamics, immediately entering the rapid growth phase (Figure 1B). The stationary phase was reached after six days and carrying capacity was estimated at 1,000,000 cells/ml. We observed small fluctuations in the cell morphology throughout the growth cycle (Figure 2). Most of the populations displayed a small increase in cell size during the rapid growth phase (days 1 to 5) and a slight reduction in cell size during the stationary phase (Figure

2A). Populations in the two experimental blocks displayed similar cell size dynamics, but through time populations in batch 1 were slightly smaller, with a 10% cell area reduction at day 8 (two-sample *t* test, $t(8) = -4.36$, $p=0.001$).

The cell shape oscillated through time and, in contrast to cell size, displayed different dynamics in each block (Figure 2B). While small fluctuations were seen in block 1, cells presented a constant decrease in aspect ratio during block 2. At the end of the experiment, cells were slightly rounder in block 2 (comparison of day 8, two-sample *t* test, $t(8) = 4.91$, $p<0.001$). Though significant, the observed variation in the cell morphology between both experimental blocks is small.

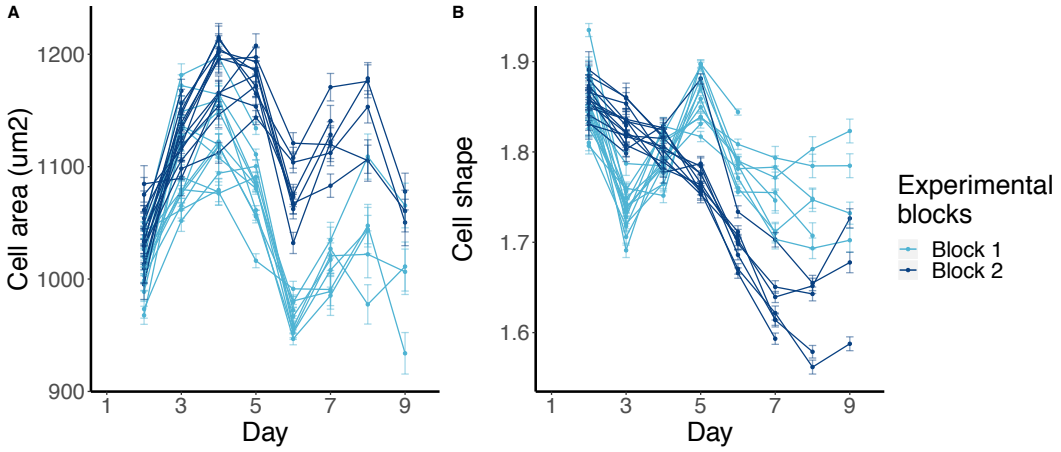

Figure 2 Morphological traits during *T. thermophila* population growth. Mean cell area (A) and mean cell aspect ratio (B) for all the populations in each day of the experiment. Each line represents on replicate population, and the error bars indicate standard errors of the mean calculated among individuals. The colors represent the two experimental blocks.

3.2. Transcriptome profiling

We identified a total of 22,080 genes expressed in the *T. thermophila* populations during growth (RNA-seq read count > 10). Principal component and clustering analyses identified two major groups (Figure 3A and Figure S3), one from populations sampled during the exponential phase (sampling points A, B and C) and a second one from populations sampled after the stationary phase was reached (sampling points D, E and G). Differential expression analyses comparing all six

sampling points identified 3,368 differentially expressed genes (Figure 3B) throughout the growth cycle. The greater the time difference between two sampling points, the larger the number of differentially expressed genes.

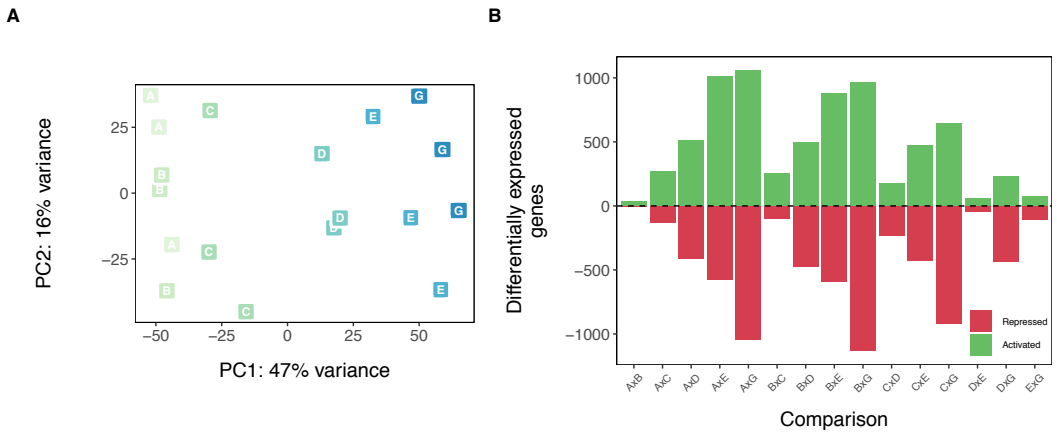

Figure 3 Transcriptome profiling during growth in *T. thermophila*. A) Principal component analysis of the transcriptomes of 18 populations based on the regularized log counts of the RNA-seq data. Each population is represented by its sampling point name (see Figure 1B). B) Differentially expressed genes in each of the 15 pairwise comparisons.

Since the largest transcriptome differences were observed between the rapid growth phase and the stationary phase, subsequent analyses were performed only with the nine pairwise comparisons that involve these two phases. When focusing on this subset, 3239 genes were differentially expressed, of which 1615 genes were overexpressed in the exponential phase, while 1588 genes were overexpressed in the stationary phase (Figure 4A). Only 36 genes were overexpressed in both exponential and stationary phases, depending on the pairwise comparison. These genes were excluded from further analyses.

Gene ontology terms are divided into three domains: molecular function (activities of the gene product at the molecular level), cellular component (cellular structures in which the gene product acts or which it is a part of), and biological process (large biological programs in which the gene product participates) (The Gene Ontology Consortium 2019). We performed GO enrichment analyses including all three domains simultaneously, and only GO terms related to molecular function or biological process were enriched in our experiment. We identified GO terms related

to energy metabolism, catalytic activities and the transport of molecules as important in the rapid growth phase (Figure 4B). For the stationary phase, processes related to the phosphorylation and deacetylation of proteins, chromatin silencing and proteolysis were overrepresented. KEGG pathway enrichment analyses indicated the importance of six pathways in the exponential phase, including the metabolism of different molecules, such amino acids and propanoate, DNA replication and synthesis of secondary metabolites (Table S1). No enriched KEGG pathway was found in the stationary phase after correction for multiple testing.

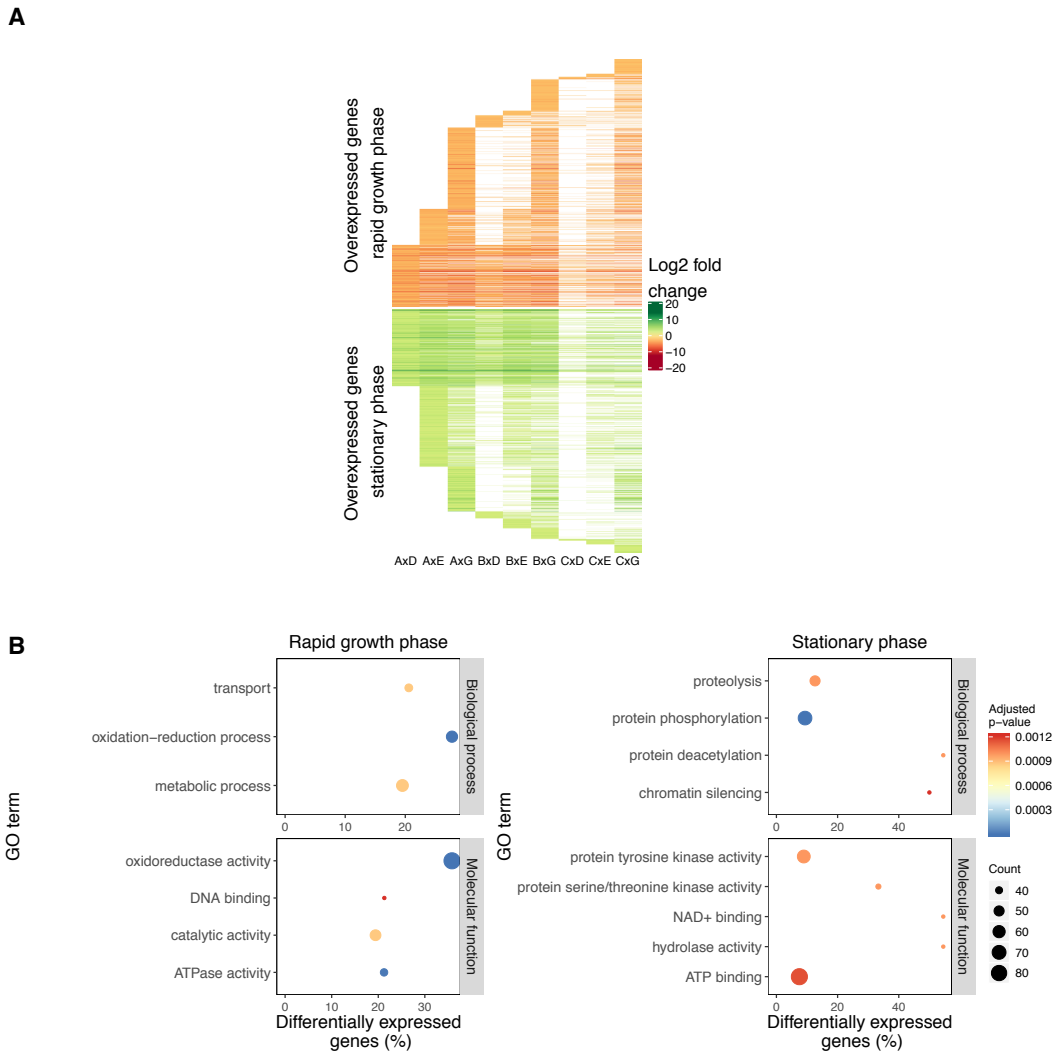

Figure 4 Transcriptome differences between the rapid growth phase and the stationary phase in *T. thermophila*. A) Heatmap of the estimated $\log_2$ fold change in gene expression. Each row represents one gene, and each column represents the estimated change in gene expression between two sampling points. The color of each cell represents the estimated fold change, and

white cells are non-significant log_2 fold changes. The names at the bottom of the columns identify the sampling points in the comparison (names are the same as in Figure 1). Adaptive shrinkage estimator (Stephens 2017) was used to shrink the log_2 fold changes for better visualization. B) Summary of the enriched GO terms in the upregulated genes at each phase, separated into the different GO classes. Size of the circles indicates number of differentially expressed genes in the category, and color shows the adjusted *p*-value (< 0.01).

3.3. H3K4me3 and H3K9ac profiling

We investigated the genome-wide distribution of the histone post-translational modifications H3K4me3 and H3K9ac by ChIP-seq, monitoring six sampling points during logistic growth. ChIP-seq reads were enriched downstream of the transcription start site (TSS) of genes, as can be seen in Figure 5A. The number of enriched regions ranged from 14,449 to 21,029 for H3K4me3 and from 15,495 to 22,884 for H3K9ac (Figure 5B). H3K4me3 peaks had an average width of 616 bp and 89 % of the peaks ranged between 116 bp and 1000 bp, while the H3K9ac peaks had an average width of 575 bp and 92 % of the peaks were between 121 bp and 1000 bp.

Three populations (population 27 from sampling point B and populations 5 and 16 from sampling point D) had a low enrichment that was indistinguishable from the control (Figure 5A), resulting in a very small number of peaks detected in both histone PTMs (Figure 5B). ChIP-seq reads from these three samples were of good quality and libraries presented similar sequencing depth to the other samples (Figure S2), which could indicate biological significance. However, since problems with chromatin isolation or immunoprecipitations cannot be excluded, we removed these three samples from the remaining analyses.

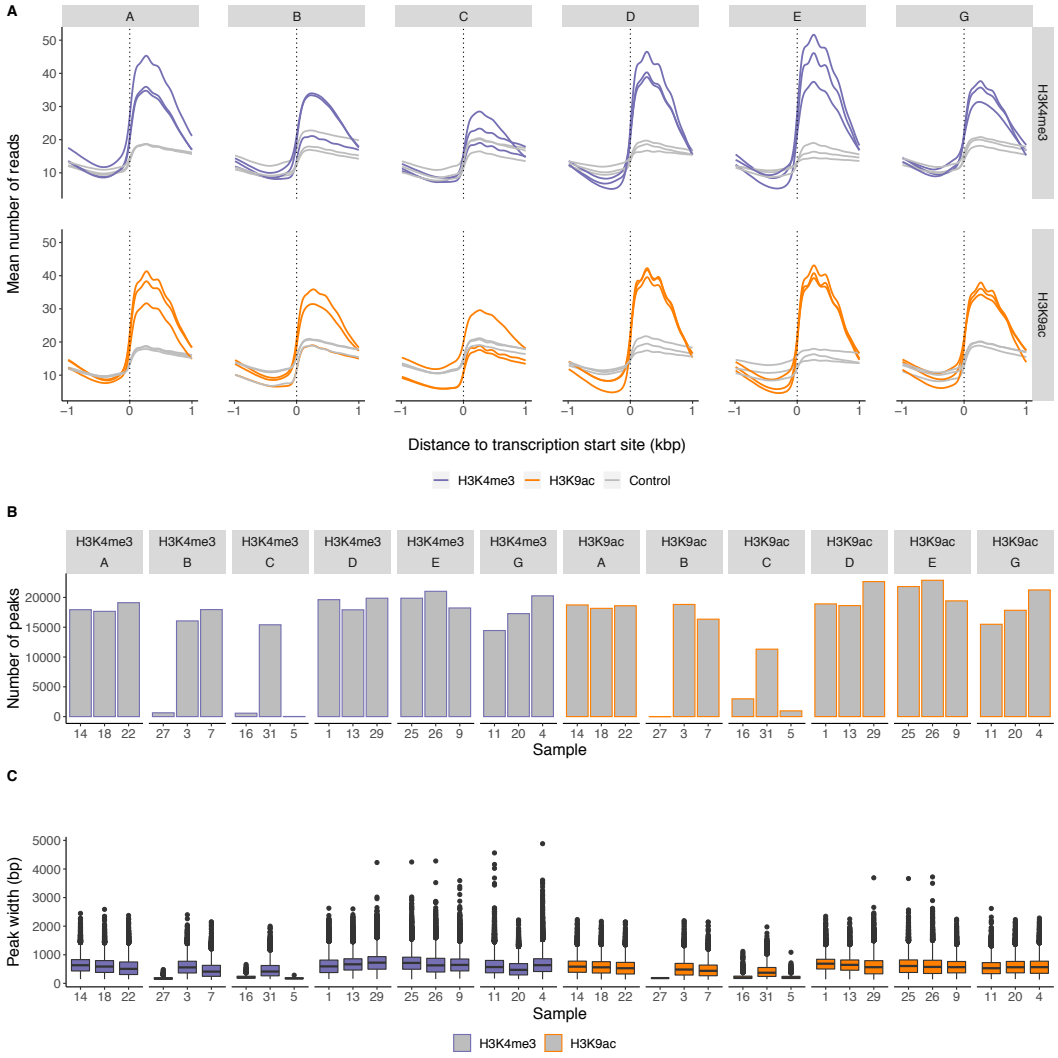

Figure 5 Genome-wide distributions of H3K4me3 and H3K9ac in *T. thermophila*. A) Plots show mean enrichment levels above background (grey) of H3K4me3 (purple) and H3K9ac (orange) for the three replicate populations in each of the six sampling points (A to G). B) Number of enriched regions (peaks) in each population for both histone PTMs. C) Boxplot of the width of the peaks in each population for both histone PTMs.

Both H3K4me3 and H3K9ac were mostly located in intragenic regions, especially within exonic regions (Figure 6A). The same pattern was observed in all sampling points across the growth period. Each peak was annotated to its closest gene, and we determined the presence of these two histone PTMs in the same gene. On average, 54 % of genes were marked with both H3K4me3 and H3K9ac, 9 % with

H3K4me3 or H3K9ac while 37 % of genes were not marked with either of these histone PTMs (Figure 6B).

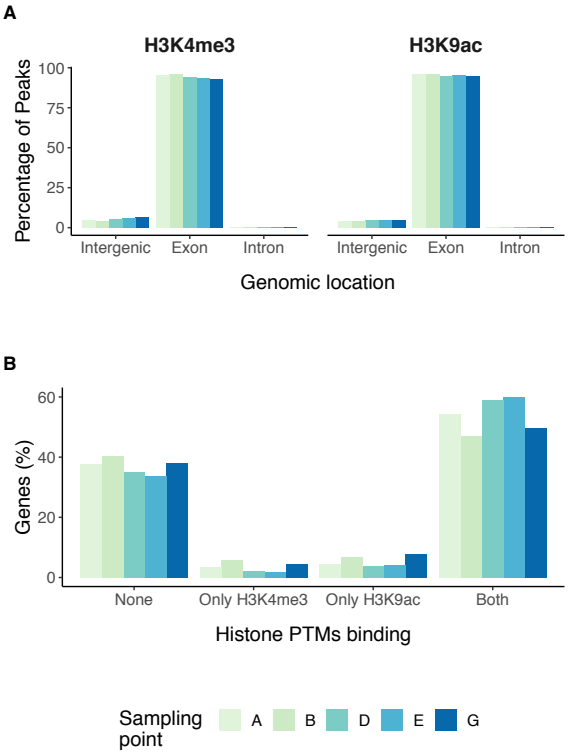

Figure 6 H3K4me3 and H3K9ac are enriched in the exons of genes in *T. thermophila*. A) Bar plot showing the percentage of peaks located in intergenic, exonic or intronic regions for both histone PTMs in each sampling point. B) Bar plot showing the percentage of genes that contained the two histone PTMs in each sampling point. In all plots, the colors of the bars indicate the sampling points as in Figure 1B.

To investigate the relationship between the presence of the histone PTMs and gene expression, we used the normalized RNA-seq data from each population to classify the genes into four levels of gene expression: no expression (rlog count < 0), low expression (0 < rlog count > 5), middle expression (5 < rlog count > 10) and high expression (rlog count > 10). We then measured the mean enrichment of ChIP-seq reads in the genes within each category. We observed that highly transcribed genes correlated with higher ChIP-seq enrichments, and this pattern was observed in all samples for both histone marks (Figure 7A).

Differential binding analyses were used to identify differences in enrichment between the rapid growth and the stationary phase for both histone PTMs. Since only

one population from sampling point C had good quality data, this sampling point had no replication and was therefore removed from the analysis. Peaks that were present in at least two replicates from each sampling point were included in these analyses, resulting in 17,354 peaks for H3K4me3 and 18,119 for H3K9ac. Only a small number of regions were identified as being differentially enriched compared to the much larger number of differentially expressed genes (Figure 7B). However, a pattern similar to the RNA-seq data was identified, with an increasing number of differentially enriched regions occurring as the time between the sampling points increases, especially in H3K4me3.

We also evaluated the correlation between changes in enrichment of the histone PTMs and changes in gene expression. We focused only on the genes that differ between the rapid growth and the stationary phase. Although the number of genes is limited, we found a small but significant correlation in eight out of twelve comparisons (Figure 7B). Similar to the previous analysis, the correlation was stronger for H3K4me3. We also analyzed the correlation between the peak width and gene expression, but no significant correlation was found. Gene ontology enrichment analyses were performed as before with the RNA-seq data by separating peaks into those present during the rapid growth phase versus those present during the stationary phase. No significant function was enriched after correction for multiple testing.

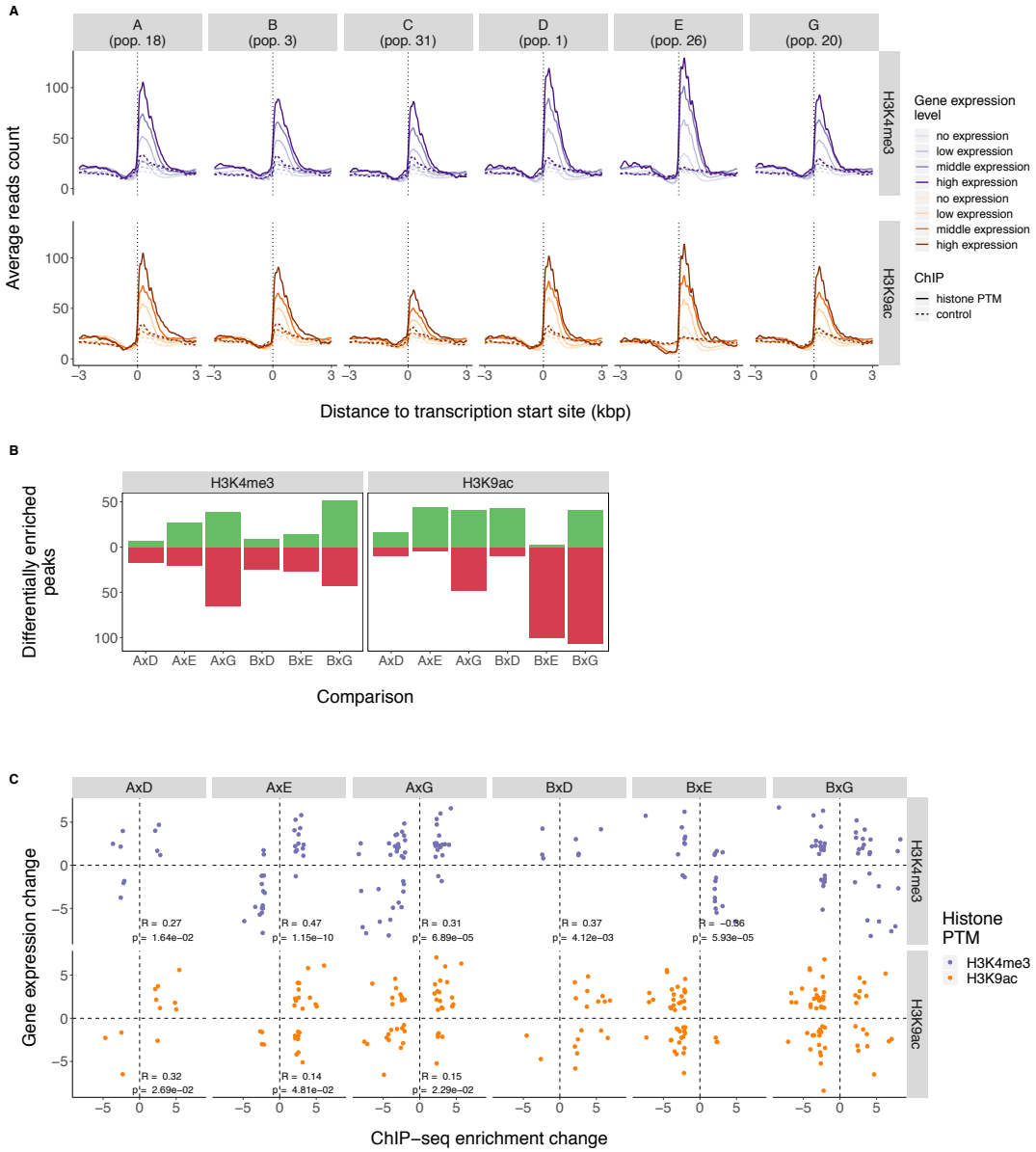

Figure 7 H3K4me3 and H3K9ac correlate with gene expression in *T. thermophila*. A) Plots show mean enrichment of ChIP-seq reads around the TSS of genes divided into four levels of gene expression. Darker colors represent higher levels of gene expression, and continuous lines represent the ChIP enrichment, while dashed lines are the controls. Letters on the top of the panels represent the population sampling point (A to G), and the numbers identify the populations. Only one replicate population from each of the six sampling points is shown, since the patterns were very similar between all replicates. B) Number of differentially enriched regions for each histone PTM. Only the six comparisons that involve populations from the rapid growth phase versus the stationary phase are shown. C) Scatterplot of the difference in significant ChIP-seq enrichment for both histone PTMs against the $\log_2$ fold change in gene

expression. The same sampling point comparisons as in plot B are shown. Spearman correlations were calculated for each comparison, and the significant correlations (*p*-value < 0.05) are displayed.

4. Discussion

In this study, we monitored cell morphology, gene expression patterns and the distribution of two histone post-translational modifications in populations of *T. thermophila* during logistic growth. We found small changes in cell morphology, but strong changes in the transcriptome of the populations. The largest differences in gene expression occurred between the rapid growth phase and the stationary phase. The two histone PTMs, H3K4me3 and H3K9ac, displayed very similar distributions across the genome, with a strong enrichment in exons. Their localization correlated with the gene expression.

4.1. Cell morphology during population growth

The populations exhibited a logistic growth pattern that was very similar across all population replicates. Small fluctuations in the cell morphology were observed during this period. Populations reached a maximum cell size in the middle of the exponential phase, which was followed by a decline that was later recovered at the stationary phase. Cells shape was also affected, with rounder cells at the end of the growth period. A previous study by Fjerdingstad et al. 2007 grew ten *T. thermophila* strains and found similar morphology dynamics during growth. Cell size is related to nutrient availability in the environment (Hellung-Larsen et al. 1993), and since cell size starts to decrease at the end of the rapid growth phase, with lower nutrient availability, we believe this factor might contribute to the observed changes in cell size during growth. However, mean cell size increases again during the stationary phase despite a continuous decrease in nutrients, indicating that other factors might also be influencing cell morphology.

4.2. Molecular basis of population growth

The transcriptome analyses revealed strong differences in gene expression between the populations sampled at the rapid growth phase and the ones at the stationary phase (Figure 3 and Figure 4). A similar pattern was detected in a study with *Bacilus subtilis*, in which large transcriptional differences between the rapid growth phase and the stationary phase were observed (Blom et al. 2011). However, this previous investigation also detected strong differences between early and late stationary phases, which were not observed in our experiment.

The functional enrichment analyses of the differentially expressed genes suggest that energy metabolism is important in the rapid growth phase, as functions related to ATP metabolism, the degradation of carbohydrates (i.e. propanoate metabolism) and amino acids (e.g. glutathione metabolism, valine, leucine and isoleucine degradation) are over expressed in this period. The gene expression analyses of Xiong et al. 2012 also found genes related to amino acid metabolism overexpressed during the growth phase, corroborating our results.

When populations enter the stationary phase, a set of genes responsible for modifying proteins through phosphorylation or acetylation is overexpressed. *T. thermophila* has a large number of kinases that are still poorly characterized, but these post-translational modifications are important in the regulation of various biological processes (Tian et al. 2014). A previous study investigating the bacteria *Rhodobacter sphareoides* during different time points of the stationary phase found small changes in the transcriptome but many changes in the proteome, also pointing to the importance of post-translational modifications of proteins in this growth stage (Bathke et al. 2019).

Besides these regulatory processes, proteolysis was also overrepresented in the stationary phase. A previous study showed that *T. thermophila* cells grown in a low nutrient medium initiate protein degradation very quickly (Jonassen and Grinde 1986), so the low nutrient conditions during the stationary phase may also trigger this response. Hydrolase activity was another biological process enriched in this period, which similarly points to the importance of catabolic processes in this stage.

4.3. Gene expression modulation during population growth

H3K4me3 and H3K9ac were highly abundant and widely distributed across the genome of *T. thermophila*. These histone PTMs were strongly enriched in downstream regions of the transcription start sites, especially in the exons (Figure 5 and Figure 6). We observe that a high proportion of genes are associated with both H3K4me3 and H3K9ac (Figure 6B). Although the distribution profile of both peaks is very similar (Figure 5A), it is difficult to infer from our data whether both histone PTMs are marking the same nucleosome. A previous study, using mass spectrometry analyses, found H3K4me3 and H3K9ac co-occurring in the same histone in *T. thermophila* (Taverna et al. 2007). Nevertheless, the mean peak width of both histone PTMs in our study was more than 500 bp, which could include more than one nucleosome in the same region, and the histone PTMs could therefore be marking different nucleosomes.

We found a positive correlation between gene expression levels and histone PTM enrichment (Figure 7A). Previous studies have found that H3K4me3 and acetylated H3 are enriched in macronuclei, the transcriptionally active nucleus of *Tetrahymena* (Vavra, Allis, and Gorovsky 1982; Strahl et al. 1999). Taken together, the results indicate that these histone PTMs mark active genes, a pattern that has also been found in other species such as the protozoan parasite *Trichomonas vaginalis* (Song et al. 2017), *Plasmodium falciparum* (Bártfai et al. 2010), *Toxoplasma gondii* (Gissot et al. 2007) and yeast (Pokholok et al. 2005). However, only few regions were differentially enriched during the growth period, in contrast to the many differentially expressed genes observed in the transcriptome analyses. This suggests that these histone PTMs are not related to the major changes in gene expression observed throughout the growth period. Other gene expression modulators may have a more significant importance, such as transcription factors, small RNAs or other histone PTMs.

In summary, we have explored the molecular basis of logistic growth in *T. thermophila*, revealing large changes between the rapid growth phase and the stationary phase. Many cell functions affected by nutrient availability change during the growth period, indicating the strong effect of the nutritional conditions of the environment in cell functioning. H3K4me3 and H3K9ac were mostly enriched downstream of TSS, marking active genes, but their role in modulating these functional changes seems to be limited. These histone PTMs might have a more

relevant regulatory role in conditions not explored in this study. Further investigations of phenotypic traits and gene expression patterns in other growth dynamics will improve our mechanistic understanding of this important biological process.

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Supplementary material

Table S1 List of enriched KEGG pathways in the overexpressed genes during the rapid growth phase.

KEGG ID	Description	Adjusted *P*-value	Gene IDs
tet00640	Propanoate metabolism	5.42E-06	TTHERM_00695650/TTHERM_00079540/TTHERM_00035180/TTHERM_00554420/TTHERM_00325580/TTHERM_00537060/TTHERM_00794540/TTHERM_00437600/TTHERM_00375130/TTHERM_00926980/TTHERM_00277470/TTHERM_00242260/TTHERM_00370710/TTHERM_00214740/TTHERM_00043870/TTHERM_00052470
tet00480	Glutathione metabolism	2.22E-05	TTHERM_00572000/TTHERM_00569200/TTHERM_00569160/TTHERM_00630450/TTHERM_00661650/TTHERM_00066 1568/TTHERM_00661570/TTHERM_00516440/TTHERM_00046110/TTHERM_00316210/TTHERM_00405450/TTHERM_00211500/TTHERM_00211510/TTHERM_00572180/TTHERM_00569210/TTHERM_00661620/TTHERM_00242370/TTHERM_00572020/TTHERM_00661630/TTHERM_00602870/TTHERM_01049360/TTHERM_00794110/TTHERM_00661640/TTHERM_00332090/TTHERM_000672289/TTHERM_00250970
tet03030	DNA replication	2.22E-05	TTHERM_00048980/TTHERM_00865240/TTHERM_000011759/TTHERM_01107420/TTHERM_00243710/TTHERM_00691170/TTHERM_00571880/TTHERM_00277550/TTHERM_00448570/TTHERM_00780750/TTHERM_00106890/TTHERM_00142290/TTHERM_00554270/TTHERM_00092850/TTHERM_00069420
tet00280	Valine, leucine and isoleucine degradation	7.40E-05	TTHERM_00530250/TTHERM_00502240/TTHERM_00052130/TTHERM_00325580/TTHERM_00686100/TTHERM_00794540/TTHERM_00088050/TTHERM_00899460/TTHERM_00926980/TTHERM_01123840/TTHERM_00277470/TTHERM_00

			214740/TTHERM_00043870
tet00630	Glyoxylate and dicarboxylate metabolism	5.49E-03	TTHERM_00529790/TTHERM_00794020/TTHERM_00030190/TTHERM_00926980/TTHERM_00318570/TTHERM_00277470/TTHERM_01141570/TTHERM_00784420/TTHERM_00043870/TTHERM_00290670
tet01110	Biosynthesis of secondary metabolites	7.27E-03	TTHERM_00529790/TTHERM_00530250/TTHERM_01207600/TTHERM_00077120/TTHERM_00079540/TTHERM_00554320/TTHERM_00417930/TTHERM_00417920/TTHERM_00325580/TTHERM_00825330/TTHERM_00486810/TTHERM_00794020/TTHERM_00794540/TTHERM_00437600/TTHERM_00661530/TTHERM_000653668/TTHERM_01014670/TTHERM_00899460/TTHERM_00974260/TTHERM_00444470/TTHERM_00346410/TTHERM_00079070/TTHERM_00030190/TTHERM_00926980/TTHERM_00112550/TTHERM_000160999/TTHERM_00859320/TTHERM_00318570/TTHERM_00794110/TTHERM_00439000/TTHERM_00268030/TTHERM_00641240/TTHERM_00277470/TTHERM_01141570/TTHERM_00189320/TTHERM_00784420/TTHERM_00242260/TTHERM_00370710/TTHERM_00043870/TTHERM_00290670

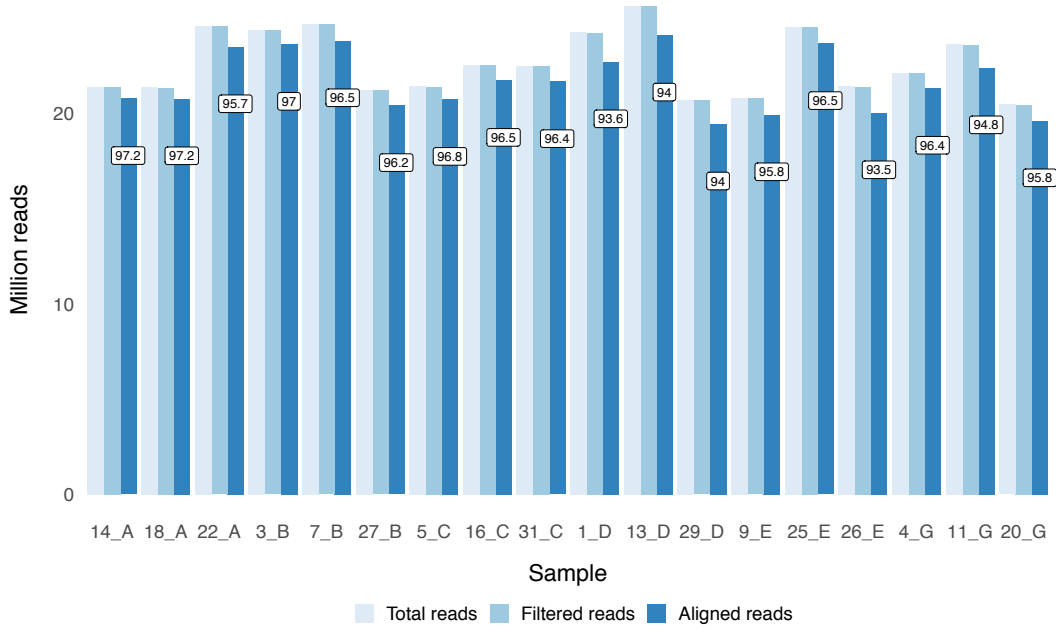

Figure S1 Summary of the quality control and alignment of the RNA-seq data. Each group of three bars represents one sample, and the sample name is composed of the number identifying the experimental population followed by the sampling point. The bars show total number of reads obtained in the sequencing, the reads that survived quality filtering and the reads that were uniquely aligned to the reference genome (Q>30). Numbers show percentage of reads that aligned to the genome.

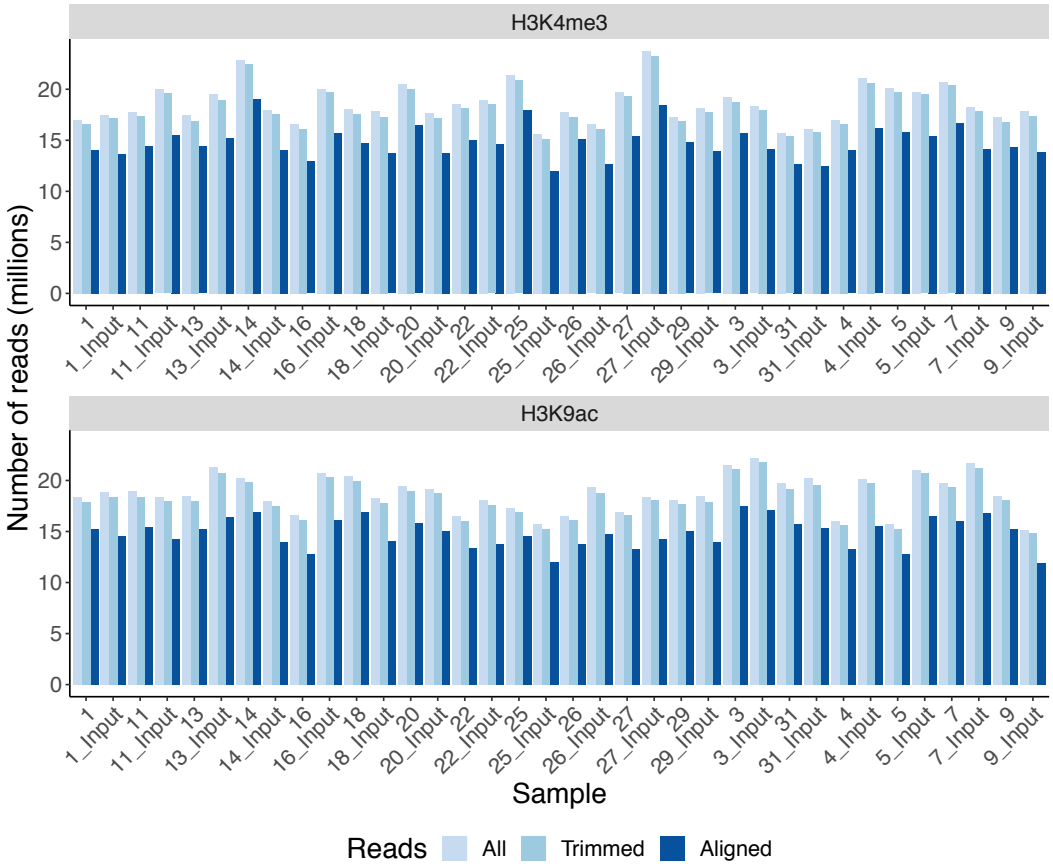

Figure S2 Summary of the quality control and alignment of the ChIP-seq data for each histone PTM separately. Each group of three bars represents one sample, and the sample name is the number identifying the experimental population. Samples called "input" are the control immunoprecipitations, performed without antibody. The bars show the total number of reads obtained in the sequencing, the reads that survived quality filtering and the reads that were uniquely aligned to the reference genome (Q>30).

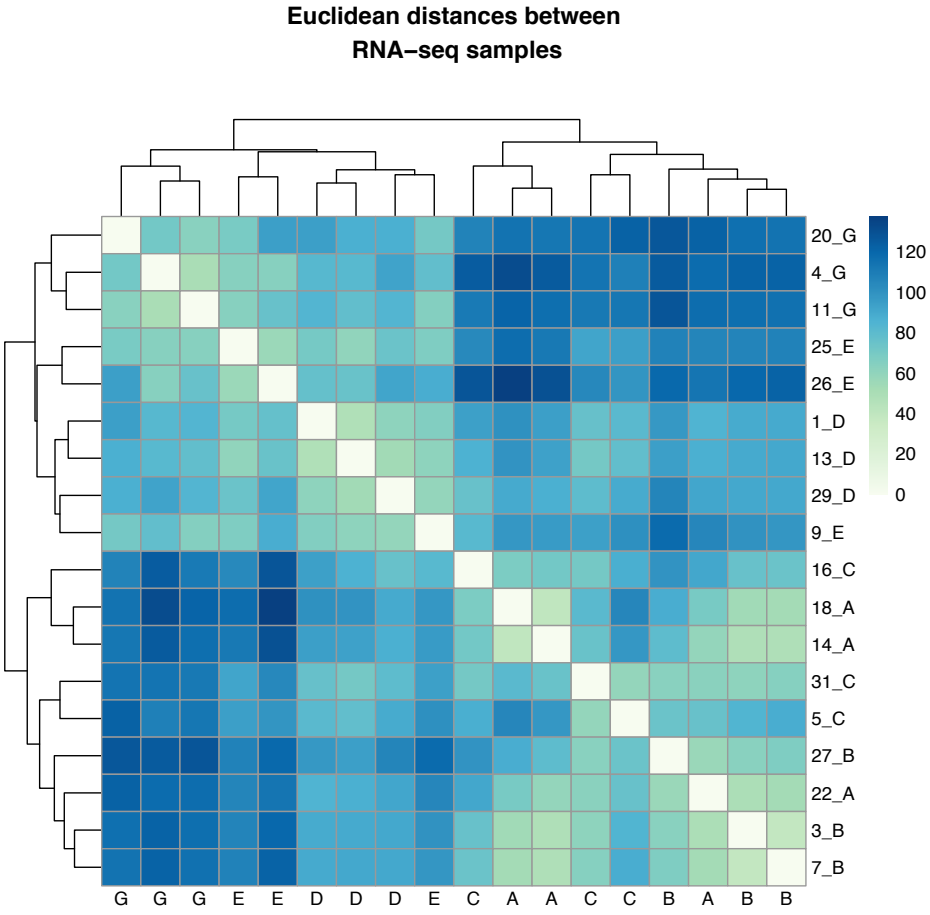

Figure S3 Heatmap of the Euclidean distances between the transcriptomes of the 18 *T. thermophila* populations sampled during the growth period. Euclidean distances were calculated based on the regularized log counts of the RNA-seq data. The row names are composed of the number identifying the experimental population followed by the sampling point. The column names are only the sampling point.

Chapter 5. General discussion

Main findings

In this thesis, I used the model organism *T. thermophila* to investigate phenotypic and molecular responses during different ecological and evolutionary processes. First, I examined populations exposed to a novel temperature close to this species' thermal limit, analyzing both cell morphology and gene expression changes in response to this important abiotic factor. The second experiment of this thesis focused on population dynamics during logistic growth, a common growth pattern in microbial species characterized by an initial period of growth in the absence of resource limitation, followed by a period of increasing resource limitation. In this experiment, besides gene expression patterns I also analyzed two histone post-translational modifications (PTMs) and their role in regulating gene expression.

I found strong effects of temperature on population dynamics and cell morphology, which are summarized in **Chapter 2**. Long lag phases were initially observed when populations grew in a novel high temperature environment for 35 generations. Maximum growth rate increased with time, while lag phase duration decreased, indicating that populations were able to adapt to this novel temperature. Smaller and rounder cells were the main morphological changes observed at 38 °C. These results confirm predictions from the temperature-size rule (Atkinson 1994), although additional studies of the role of cell shape in response to temperature are needed, in order to further understand the observed patterns.

Besides analyzing responses to the high temperature environment, I also monitored populations that moved back to the original temperature of 20 °C after growing for two batches at 38 °C. The aim of this second part of the experiment was to verify if any costs related to survival at 38 °C were present, using the populations in the first batch at 20 °C as a reference. Although longer lag phases and slightly smaller cell sizes were observed, most of the traits returned to the range observed in the ancestral populations, which indicates a large role of phenotypic plasticity in the temperature response of this species.

The same populations in the temperature experiment were then employed in the transcriptome profiling in **Chapter 3**. Extensive changes in gene expression

patterns were observed when populations moved to 38 °C, with 6,057 genes differentially expressed. Functional enrichment analyses revealed the downregulation of genes responsible for carbohydrate metabolism and proteolysis, suggesting that energy balance and metabolism repression are important components of the heat stress response in this species.

The transcriptome analyses of the populations that returned to 20 °C after two batches at 38 °C were performed both within and between generations. The within response was measured after only one hour of exposure to 20 °C and revealed substantial changes in gene expression, another indication that phenotypic plasticity plays an important role in the temperature response. The between generation effect was even stronger, and the transcriptomes of the populations at 20 °C in batches 4 and 5 were very similar to the ones of the populations in batch 1.

In **Chapter 4**, the experiment analyzing populations of *T. thermophila* across different time points of logistic growth revealed small changes at the phenotypic level, but extensive differences at the transcriptome level. The majority of changes in gene expression were found between the rapid growth phase and the stationary phase, with genes related to energy metabolism upregulated in the rapid growth phase, and genes responsible for protein post-translational modifications upregulated in the stationary period.

To further investigate gene expression in this species, two histone PTMs were monitored in this study, trimethylation of histone H3 at lysine 4 (H3K4me3) and acetylation of histone H3 at lysine 9 (H3K9ac), which have putative transcriptional regulation activity. Both histone PTMs displayed very similar genome-wide distribution patterns, with enrichment in exonic regions and marking active genes. We found little correlation between the changes in gene expression and the changes in enrichment of histone PTMs, which indicates that other mechanisms are responsible for the observed transcriptional changes, such as different histone PTMs or other transcriptional modulators like transcription factors or small RNAs.

Morphological responses to the temperature in *T. thermophila*

Cell morphology of *T. thermophila* was monitored in all experiments of this thesis. While the growth experiment (Chapter 4) was performed at 30 °C, the temperature experiment used two conditions, 20 °C and 38 °C (Chapter 2). The

same culture conditions were used, and I therefore compared the dynamics of cell size and cell shape in both experiments, which can be seen in Figure 1.

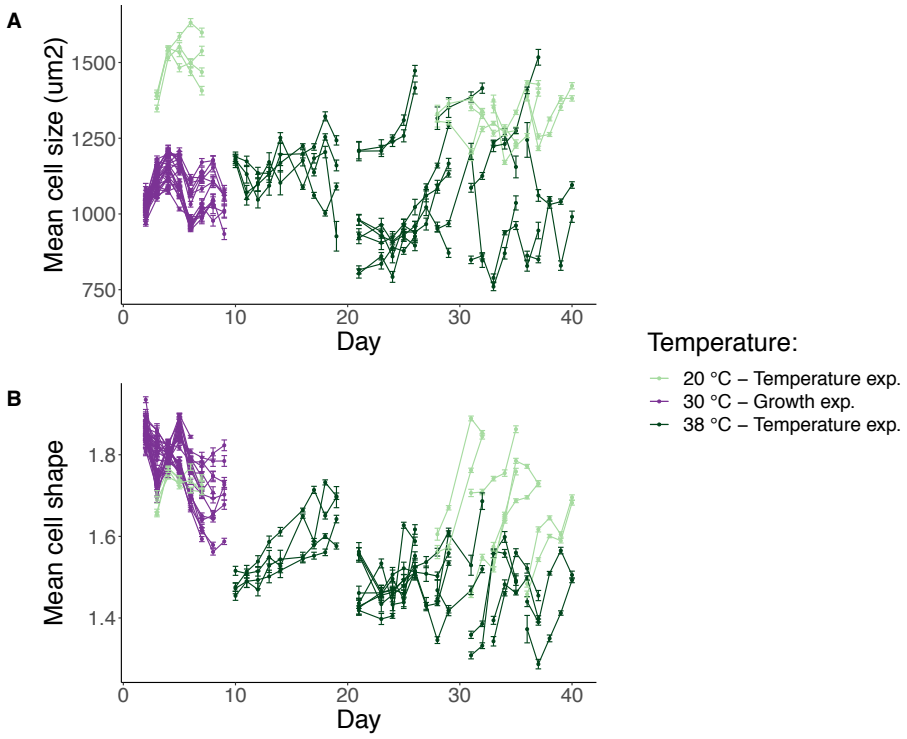

Figure 1 Cell morphology dynamics in *T. thermophila* grown at different temperatures. Data was collected in two experiments (see methods in Chapters 2 and 4) that used the same *T. thermophila* strain and identical growth conditions. Mean cell size and (A) mean cell shape (B) of each population. Each line represents on replicate population, and the error bars indicate standard errors of the mean calculated among individuals.

Each morphological trait displayed different dynamics in relation to temperature. While cell area at 30 °C was strongly reduced in comparison to 20 °C and presented similar range to the cell area at 38 °C (Figure 1A), cell shape displayed the opposite pattern, with similar cell shapes at 20 °C and 30 °C and rounder cells (smaller values) only at 38 °C. The different patterns observed are evidence that these morphological traits are not correlated and respond differently to temperature and possibly to other environmental factors. Investigating the stability of these patterns with longer experiments would also be important, since cell size responses could be faster than changes in cell shape.

Gene expression patterns in *T. thermophila*

Transcriptome profiling through RNA-seq proved to be a suitable method for exploring gene expression in this species. While the *T. thermophila* genome that was used as a reference in all analyses (genome release 2014, available at Tetrahymena Genome Database, Stover et al. 2012) contains 26,996 genes, the transcriptome analyses detected 23,680 genes expressed during the temperature experiment and 22,080 during the growth experiment. The overlap between both experiments was very large (21,703 genes, 90.2 % overlap), confirming the broad detection range of RNA-seq. We also found a very high correlation between replicate populations in the growth experiment, demonstrating the accuracy of this method (not shown). The correlation between replicate populations in the temperature experiment was not as high, probably due to the longer duration of the experiment and the larger differences in population dynamics through time.

It would be interesting to compare the transcriptome data from both experiments, which would result in a dataset with populations grown across three different temperatures. A simple comparison of these samples with principal component analyses revealed large differences between the two experiments (Figure 2), and hierarchical clustering analyses also showed the strongest differences between the experiments (not shown). Great caution is required when comparing samples from separate experiments, since differences in protocols can generate significant biases in sequencing data. Each experiment used a different library preparation method and the samples were sequenced in different sequencing platforms. There are also differences in read length and in library sequencing depth. Although it is still possible to compare samples with such differences, as shown in previous reports (Li et al. 2014; Su et al. 2014), these analyses are not as simple and would require careful modeling of the gene expression changes, taking into account these methodological differences.

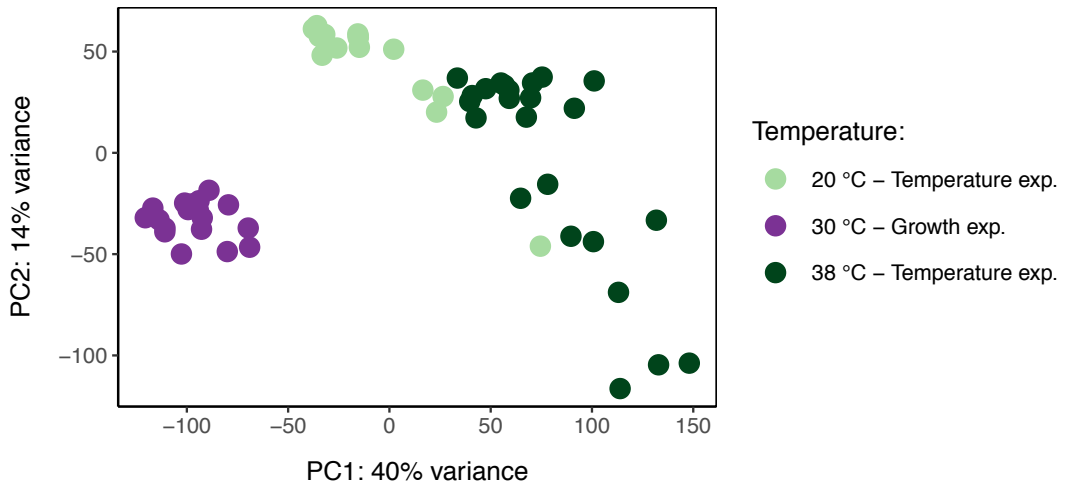

Figure 2 Principal component analyses of the RNA-seq samples from the growth and temperature experiments. Analyses are based on regularized log counts. Each point represent one population the colors indicate the temperature in which the population was grown.

Gene expression analyses during the different stages of logistic growth showed that significant changes occur between the rapid growth phase and the stationary phase. These results highlight the importance of comparing populations sampled in the same growth period when analyzing transcriptomes of *T. thermophila*. In the temperature experiment, the populations were always sampled at the stationary phase, reducing the potential noise that could be introduced by comparing different growth stages. A previous study found a significant increase in the average mRNA content per cell during the growth cycle of a marine *Betaproteobacteria* (Gifford et al. 2016), which could also lead to biases in differential expression analyses. Taken together, these observations indicate that growth stage should be carefully considered when designing transcriptome studies.

Challenges in the study of *T. thermophila*

Although *T. thermophila* is considered a model organism and has many valuable resources, its use in research still presents many challenges. The nuclear dimorphism of this species is an important feature in many studies, as discussed in the general introduction of this thesis, but it also poses challenges to its use in laboratory experiments. The two nuclei inside each cell need to be separated before

techniques like genome sequencing or chromatin immunoprecipitation can be performed, and nuclei separation is usually long and requires large volumes of culture (e.g., Allen et al. 1983; Melody T. Sweet and C. David Allis 2010). In this project, I spent a few months optimizing a nuclei isolation protocol (Sweet and Allis 2006) for its use with the smaller culture volumes that are usually employed in ecological and evolutionary experiments. Although the protocol was improved, nuclei separation still posed limitations to experimentation. Cells sampled during an experiment can only be frozen after nuclei separation, since the nuclei membranes are disrupted upon freezing and they have to be intact for nuclei separation. This increased the time required for sampling and significantly limited the number of populations that could be sampled in parallel during experiments.

The transcriptome analyses in this thesis revealed many genes with large changes in their expression but with no known function, a common outcome when studying many species (Pavey et al. 2012). Out of the 26.996 genes in *T. thermophila*, only a small fraction presents a functional annotation (23 % have a gene ontology term and 7% have a Kyoto Encyclopedia of Genes and Genomes pathway). Many biological mechanisms important during population growth and temperature stress are probably not identified in this study due to lack of gene annotation. New methods are being developed to better annotate genomes and new studies with *T. thermophila* will surely increase the knowledge about its genes and their functions. Analyzing the data generated during this project with an improved genome annotation in the future might be valuable and could reveal new insights into these biological processes.

Future directions

All model systems present their unique benefits and disadvantages, and although I faced challenges with the use of *T. thermophila* during my thesis, I still believe this species is a valuable system for ecological and evolutionary investigations. The questions I addressed during my PhD project could be further explored in many ways. I highlight here some ideas that I find most appealing.

For the temperature experiment in Chapters 2 and 3, I believe a better integration of the cell morphology and gene expression data would be important. A few studies in the 1980s have identified genes in *T. thermophila* with an effect in cell

size and shape (see references in the review of Wloga and Frankel 2012), so these could be interesting candidates for initial analyses. Looking for homolog genes from other model organisms such as *Paramecium* might also help identifying relevant genes.

During the temperature experiment, besides sampling cells for RNA-seq, I also isolated macronuclei from all 40 populations. The original goal was to investigate histone PTMs dynamics and relate them to gene expression changes, similar to the analyses performed with the data from the growth experiment in Chapter 4. Due to time limitations it was not possible to perform these ChIP experiments during my PhD project. These samples could still be used for investigation of histone PTMs, or also for the analyses of histone variants such as H2A.Z, which has a role in response to environmental factors (Talbert and Henikoff 2014). Another possibility would be to use these macronuclei for genome sequencing and to evaluate single nucleotide polymorphism and other mutations that might have occurred in these populations during the experiment, better understanding the role of mutations in these dynamics and connecting them to the gene expression changes.

The transcriptome analyses revealed many genes with potential functions in temperature response and in specific growth stages. Although interesting, these results opened many more questions about the actual role of these genes in *T. thermophila*. It would be extremely valuable to perform functional analyses of some of the genes and pathways identified in this study, which would allow us to gain a more mechanistic understanding of these processes at the cellular level. These studies require complex experiments, such as the development of gene knockouts followed by functional assays, but they are possible with this species and crucial in advancing the understanding of cell functioning. Gene knockouts were used, for example, to clarify the function of small RNAs in genome rearrangements (Mochizuki et al. 2002) and to identify the separate roles of two dynein proteins in *T. thermophila* (Lee et al. 1999). Similar approaches would be interesting, for example, with the upregulated genes at 38 °C that presented very large fold changes across the entire experiment and have unknown functions.

T. thermophila was grown in axenic conditions during all the experiments reported here, which greatly reduces the variation in environmental conditions between replicates and also facilitates the molecular analyses since there is no nucleic acid contamination from other species. However, in the natural environment *T. thermophila* usually inhabits freshwater ponds with a great diversity of organisms,

feeding on bacteria and competing with similar protists for different resources. Better understanding the processes explored here, such as morphological and transcriptomic responses to temperature, in a more complex environment within natural or assembled communities would be an exciting project and an interesting continuation to these studies. More natural conditions could also be helpful in predicting general responses of ciliates to environmental factors.

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CURRICULUM VITAE

Vanessa Weber de Melo

EDUCATION

01/2015 – present Zurich, Switzerland	**PhD in Evolutionary Biology** University of Zurich
09/2012 – 08/2014 Bern, Switzerland	**Master in Ecology and Evolution** University of Bern
04/2007 – 01/2012 Santa Maria, Brazil	**Bachelor in Biology** Federal University of Santa Maria

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RESEARCH EXPERIENCE

Doctoral thesis
01/2015 – present

Predictive Ecology group
Prof. Owen Petchey
University of Zurich

Phenotype and transcriptome responses during growth and temperature stress in the ciliate *Tetrahymena thermophila*

- Designed and conducted multiple experiments
- Characterized gene expression patterns and histone modification distribution in growth cycles using RNA-seq and ChIP-seq
- Identified phenotypic effects and relevant genes for temperature response using RNA-seq

Master thesis
09/2012 – 08/2014

Computational and population genetics group
Prof. Gerald Heckel
University of Bern

Spatiotemporal dynamics of Puumala hantavirus associated with its rodent host, *Myodes glareolus*

- Participated in two vole sampling excursions
- Generated and curated genotype dataset of voles
- Identified spatial and temporal coevolution patterns between host and virus

Bachelor thesis
03/2010 – 12/2011

Genetic Diversity Laboratory
Prof. Marlise L. Bartholomei-Santos
Federal University of Santa Maria

Reliability of COI sequences of Decapoda crustaceans in Genbank

- DNA extraction of freshwater crustaceans
- Amplification and sequencing of COI gene
- Retrieved COI sequences available on Genbank for the Decapoda group
- Performed quality controls on COI sequences

Internship
09/2009 – 02/2010

Dr. Lorenzo Brusetti
Free University of Bozen

Structure of rhizobacterial communities in an apple orchard under different nitrogen amendments

- Isolated DNA from soil bacterial communities
- Performed DGGE analysis and sequenced isolated DNA
- Identified species present in soil communities

TEACHING

Spring semester
2017/2018
Zurich, Switzerland

Data Analysis in Biology course, University of Zurich

- Revised and edited lectures content and assessments
- Teaching assistance in practical lectures
- Exam supervision

AWARDS, HONORS AND SCHOLARSHIPS

01/2014
Geneva, Switzerland

Best scientific talk
Biology 14

12/2011
Santa Maria, Brazil

Best abstract – Genetics, molecular biology and biochemistry
III Animal Biodiversity Symposium

04/2010 – 12/2011
Santa Maria, Brazil

Scholarship from Research Incentive Fund, Federal University of Santa Maria
Research project “Investigation of genetic distances between *Trichodactylus* crabs (Crustacea: Decapoda: Brachyura) from Rio Grande do Sul”

01/2008 – 12/2008 Santa Maria, Brazil	**Scholarship from Tutorial Education Program, Federal University of Santa Maria** Research, education and outreach project

OUTREACH AND SERVICE

04/2018 – 05/2019 Zurich, Switzerland	**Irchel Nature Trail project,** University of Zurich • Developed content for science boards and website
09/2016 – 09/2018 Zurich, Switzerland	**PhD students representative** Steering committee of the University Research Priority Program "Evolution in Action" University of Zurich
01/2016 – 07/2017 Zurich, Switzerland	**PhD students representative** Search committee for Assistant Professor of Ecological Modeling Faculty of Science, University of Zurich

CONFERENCES

2019 Zurich, Switzerland	Oral presentation **Temperature adaptation in the ciliate *Tetrahymena thermophila*** Biology19
2018 Heidelberg, Germany	Poster presentation **Temperature adaptation in the ciliate *Tetrahymena thermophila*** EMBO workshop – Experimental approaches to evolution and ecology using yeast and other model systems
2018 Neuchatel, Switzerland	Poster presentation **Transcriptome and chromatin dynamics during population growth of *Tetrahymena thermophila*** Biology18
2016 Liverpool, England	Poster presentation **Temperature effect on population dynamics of the ciliate *Tetrahymena thermophila***

	Annual meeting of the British Ecological Society
2015 Edinburgh, Scotland	Oral presentation **The role of epigenetics in adaptive processes in *Tetrahymena thermophila*** Annual meeting of the British Ecological Society
2014 Geneva, Switzerland	Oral presentation **Spatiotemporal dynamics of Puumala hantavirus associated with its rodent host, *Myodes glareolus*** Biology 14
2011 Santa Maria, Brazil	Oral presentation **Evaluation of the reliability of COI sequences of Decapoda crustaceans in Genbank** III Animal Biodiversity Symposium
2010 Águas de Lindóia, Brazil	Poster presentation **Are "COI-like" sequences in Genbank a problem for Decapoda crustaceans?** Genetics Brazilian Congress

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PUBLICATIONS

Accepted articles

Saxenhofer, M., **Weber de Melo, V**., Ulrich, R.G., and Heckel, G. (2017). Revised time scales of RNA virus evolution based on spatial information. Proc. R. Soc. B Biol. Sci. *284*, 20170857.

Weber de Melo, V., Sheikh Ali, H., Freise, J., Kühnert, D., Essbauer, S., Mertens, M., Wanka, K.M., Drewes, S., Ulrich, R.G., and Heckel, G. (2015). Spatiotemporal dynamics of Puumala hantavirus associated with its rodent host, *Myodes glareolus*. Evol. Appl. *8*, 545–559.

Ali, H.S., Drewes, S., **Weber de Melo, V**., Schlegel, M., Freise, J., Groschup, M.H., Heckel, G., and Ulrich, R.G. (2015). Complete genome of a Puumala virus strain from Central Europe. Virus Genes *50*, 292–298.

Under review

Weber de Melo, V., Petchey, O. Phenotypic responses to temperature in the ciliate *Tetrahymena thermophila*. Under review in Ecology and Evolution.